DÉVELOPPEMENT D'UN VACCIN VIVANT *SMALL-COLONY VARIANT* CONTRE LES INFECTIONS INTRAMAMMAIRES À *STAPHYLOCOCCUS AUREUS* ET RÔLE DU SYSTÈME SENSEUR-RÉGULATEUR GRAXRS-VRAFG DANS SON ATTÉNUATION

par

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À ma fille, Léonie

Apprendre à penser, c'est apprendre à être libre.

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SOMMAIRE

Staphylococcus aureus est un pathogène majeur d'importance humaine et animale, capable de provoquer à la fois des infections aigues pouvant entraîner une haute morbidité et des infections chroniques particulièrement difficiles à traiter. Parmi les facteurs qui peuvent expliquer l'échec des antibiothérapies et sa tendance à causer ces infections chroniques, beaucoup ont pointé le caractère dynamique de sa pathogénèse, et l'abondance de facteurs de virulence dédiés à affaiblir, désorganiser et éviter la réponse immunitaire. *S. aureus* se pose ainsi comme un commensal-opportuniste particulièrement adapté à l'environnement de l'hôte. Dans le cas de la mammite bovine, un problème majeur pour l'industrie laitière au Canada, *S. aureus* est l'agent étiologique responsable de la plupart des infections intramammaires (IIMs) cliniques, mais aussi sous-cliniques, une forme de la maladie plus difficile à percevoir et qui persiste parfois plusieurs mois, générant ainsi des lésions tissulaires conduisant à la diminution de la production et de la qualité du lait. L'échec des traitements antibiotiques conventionnels pour traiter efficacement la maladie a finalement stimulé, lors des dernières décennies, la recherche de nouvelles stratégies pour combattre le problème, notamment par le développement de vaccins pour protéger efficacement la glande mammaire contre ces infections. On sait que le phénotype *small-colony variant* (SCV) de *S. aureus*, associé couramment aux infections chroniques et à l'inefficacité de certains antibiotiques chez l'humain, a déjà été isolé lors de cas de mammites bovines, et son profil d'expression de gènes de virulence facilitant la persistance dans l'hôte pourrait refléter de façon assez analogue le profil d'expression des souches de *S. aureus* responsables des IIMs persistantes.

Cette étude prend ainsi pour prétexte les caractéristiques particulières des SCVs de *S. aureus* dans les infections persistantes pour tirer avantage de ce phénotype en l'utilisant comme souche vivante atténuée pour l'obtention de réponses immunitaires spécifiques, fortes et balancées vers la médiation cellulaire contre *S. aureus*. Une souche SCV génétiquement stable, obtenue par la délétion du gène *hemB* et une atténuation supplémentaire dans le gène *vraG*, avait été obtenue

lors de mes précédent travaux de maîtrise, et avait montré une forte atténuation dans des modèles *in vitro* et *in vivo*. L'avantage immunogène supérieur de la forme vivante du vaccin SCV atténué sur des formulations vaccinales sous-unitaires et utilisant des bactéries inactivées a pu être examiné et établi dans cette présente étude. En outre, dans l'optique de mieux comprendre les bases moléculaires de sa grande atténuation *in vivo*, cette étude dresse également une caractérisation phénotypique et transcriptomique de la souche SCV Δ*hemB*Δ*vraG* utilisée dans notre vaccin. Notamment, nous avons identifié quelques altérations dans son profil d'expression de gènes associés à la résistance à différents stress et molécules antimicrobiennes de l'hôte. Cette étude aura ainsi permis de mettre en lumière l'importance du système de détection et de résistance aux peptides cationiques GraXRS-VraFG dans l'augmentation de la persistance et de la réponse au stress de l'hôte permise par le phénotype SCV.

Mots clés : *Staphylococcus aureus*; Mammite bovine; vaccination; *small-colony* variant; vaccin atténué; peptides cationiques antimicrobiens; résistance aux antibiotiques; virulence

TABLE DES MATIERES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ABBRÉVIATIONS

ADN	Acide désoxyribonucléique
AIP	*Autoinducing peptide*
APC	*Antigen presenting cell*
CAMP	*Cationic antimicrobial peptide*
CCS	Compte de Cellules Somatiques
CFU	*Colony-forming unit*
Clf	*Clumping factor*
CMH	Complexe majeur d'histocompatibilité
CMI	Concentration minimale inhibitrice
CNA	*Collagen adhesin*
CNS	*Coagulase Negative Staphylococci*
CP	*Capsular polysaccharide*
CPA	Cellule présentatrice d'antigène
CTL	*Cytotoxic T lymphocyte*
DEG	*Differentially expressed gene*
DO	Densité optique
ELISA	*Enzyme-linked immunosorbant assay*

FgBP	*Fibrinogen-binding protein*
FnBP	*Fibronectin-binding protein*
Hla	Hemolysine-α
ID	Intra-dermal ou intradermique (voie de vaccination)
IgG	Immunoglobuline G
IgA	Immunoglobuline A
IgM	Immunoglobuline M
IL	Interleukine
IMam	Intramammaire (voie de vaccination)
IMC	Immunité à médiation cellulaire
IM	Intramusculaire
IIM	Infection intramammaire
INF-γ	Interféron gamma
LTA	*lipoteichoic acid*
MB	Mammite bovine
MIC	*Minimal inhibitory concentration*
NAS	*Non-aureus staphylococci*
OMV	*outer membrane vesicle*
PAMP	*pathogen-associated-molecular-pattern*

PBS	*Phosphate buffered saline*
PCR	*Polymerase chain reaction*
PIA	*Polysaccharide intercellular adhesin*
PMN	*Polymorphonuclear neutrophil*
PNAG	Poly-N-acetylglucosamine
PRR	*Pattern recognition receptor*
RT-PCR	*Reverse transcription polymerase chain reaction*
SAAC	*Slime associated antigenic complex*
SCC	*Somatic cell count*
SCV	*Small-colony variant*
SeC	*Staphylococcal enterotoxin* C
SI	Système immunitaire
TCS	*Two-component system*
Th-1	Lymphocyte (ou voie) t-helper de type 1
Th-2	Lymphocyte (ou voie) t-helper de type 2
Th-17	Lymphocyte (ou voie) t-helper de type 17
TSST-1	*Toxic shock syndrome toxin* 1
UFC	Unité formatrice de colonie
VISA	*Vancomycin Intermediate Staphylococcus aureus*

CHAPITRE 1

INTRODUCTION

1.1 La mammite bovine

1.1.1 Portrait général et manifestations cliniques

La mammite est la maladie la plus fréquente chez les vaches laitières et dont les effets sur le bien-être des animaux et la rentabilité des fermes laitières sont les plus néfastes. La mammite, qui par définition correspond à une inflammation de la glande mammaire, est le plus souvent due à une infection bactérienne, ou infection intramammaire (IIM). Une telle inflammation causée par des levures (*Candida*), des algues microscopiques, ou suite à un traumatisme du pis ou à des désordres physiologiques existent également, mais celles-ci sont beaucoup plus rares.

L'infection bactérienne de la glande mammaire peut se manifester en formes cliniques se classant selon la sévérité des symptômes; de la présence de grumeaux dans le lait, à l'enflure et la rougeur de la glande, jusqu'au choc toxique (fièvre, perte d'appétit, faiblesse de la vache et parfois, mort de l'animal). Une IIM peut également se manifester par une absence totale de signes macroscopiques visibles de la maladie, sous la forme d'une infection sous-clinique, qui n'est détectable que par la présence de microorganismes pathogènes dans le lait ou par une élévation anormale du compte de cellules somatiques (CCS) (> 200 000 cellules/mL) dans celui-ci (Contreras and Rodriguez, 2011). Le caractère clinique ou non de l'IIM est principalement influencé par le genre et l'espèce du pathogène responsable (Zadoks et al., 2011). Les bactéries coliformes sont souvent associées à des mammites aigües accompagnées de symptômes cliniques. Ces mammites à coliformes sont la plupart du temps contractées dans l'environnement immédiat de la vache, et causées par des espèces telles qu'*Escherichia coli*, *Klebsiella*, *Enterobacter*, et *Citrobacter* spp. (Hogan et Smith, 2003). On parle de mammite

environnementale pour décrire ce type d'IIM non contagieuse. Des pathogènes contagieux, tels que *Mycoplasma bovis*, *Streptococcus agalactiae, Staphylococcus aureus* et plusieurs staphylocoques non-aureus (NAS : *non-aureus staphylococci*) sont également couramment responsables d'IIMs (Murai et al., 2014). Ces infections se transmettent la plupart du temps pendant la traite, de vaches en vaches, par l'intermédiaire de l'équipement de traite contaminé ou des mains souillées du personnel. La grande majorité des cas d'IIM dans les troupeaux canadiens survient sous la forme sous-clinique, et le pathogène contagieux *S. aureus* est un contributeur important de celle-ci (Reyher et al., 2011).

1.1.2 Importance économique

Malgré de grands progrès dans la compréhension des interactions hôte-pathogène, l'utilisation de nouvelles pratiques de gestion agricole et le développement de nouveaux outils de traitement et de prévention, la mammite bovine reste à ce jour la maladie ayant le plus grand impact économique pour l'industrie laitière (Aghamohammadi et al., 2018; Ruegg, 2017). En incluant les coûts déboursés pour les mesures préventives, ainsi que ceux engendrés par les formes cliniques et sous-cliniques de la maladie, il a été estimé récemment que le coût moyen de la mammite pour les producteurs laitier canadiens serait d'environ 66 178 $/an pour un troupeau de 100 vaches (Aghamohammadi et al., 2018). Ce montant équivaudrait donc à environ 662 $ par vache sur une période d'un an, un chiffre des plus déstabilisant. Les IMIs sous-cliniques sont donc responsables de la plupart des problèmes économiques associés à la mammite : celles-ci représentent également la majorité des pertes totales engendrées pour les producteurs laitiers (Petrovski et al., 2006; Shim et al., 2004). Ces pertes monétaires seraient liées notamment à la perte du lait contaminé, la baisse de production des vaches infectées, aux coûts des traitements antibiotiques, aux frais vétérinaires ainsi qu'à la période de retrait des vaches traitées aux antibiotiques (Aghamohammadi et al., 2018). La répartition approximative des coûts moyens engendrés par chacun de ces composantes selon le type de mammite au Canada est détaillée au tableau 1.1.

Tableau 1.1. Répartition approximative des composantes des coûts annuels associés à la mammite au Canada

Composante	Mammite clinique (19 889$/100 vaches)	Mammite sous-clinique (34 859$/100 vaches)
Médicaments	2 %	-
Diagnostique	<1 %	-
Main d'œuvre	3 %	-
Lait jeté	11 %	<1 %
Perte de production	34 %	72 %
Réforme et mortalité	48 %	25 %

(résumé tiré des données de Aghamohammadi et al., 2018).

1.2 *Staphylococcus aureus*

1.2.1 Importance dans les infections intramammaires

Le pathogène contagieux *Staphylococcus aureus* (*S. aureus*) est l'espèce la plus souvent isolée dans le contexte des IIMs au Canada (Reyher et al., 2011). Bien que pouvant induire des mammites cliniques, il est majoritairement responsable de mammites sous-cliniques (Leitner et al., 2011; Sutra and Poutrel, 1994). Comme discuté plus avant, ces infections diminuent la qualité du lait et constituent un réservoir de transmission au sein du troupeau. Dans les mammites contagieuses à *S. aureus*, le lait provenant de vaches infectées est la principale source d'infection. La transmission des organismes à des vaches et des quartiers sains survient ainsi surtout pendant la traite (Olde Riekerink et al., 2008), mais peut également avoir lieu à d'autres moments. Cette transmission est donc ainsi extrêmement liée à la qualité de la régie de traite, des procédures de désinfection des trayons et à la propreté de l'équipement de traite utilisé de vaches en vaches.

1.2.1.1 Diagnostic

Malheureusement, le caractère sous-clinique des mammites à *S. aureus* complique le diagnostic de terrain; la plupart du temps, elles passent complètement inaperçues. Ainsi, chez les vaches laitières, les mammites à *S. aureus* s'expriment le plus souvent seulement par l'élévation des CCS dans le lait, principalement liée à un afflux des neutrophiles (ou PMNs) dans la glande (Van Oostveldt et al., 2001). Cet important indicateur de la santé du pis est souvent le seul indice disponible pour déceler ces infections, et est remarqué très à retardement par les producteurs, lorsque plusieurs vaches sont infectées dans un troupeau. Pour l'instant, ce n'est que par l'analyse bactériologique et le compte exhaustif des CCS du lait que l'on peut clairement identifier les vaches ou les quartiers sous-cliniquement infectés par *S. aureus*.

1.2.1.2 Efficacité des traitements curatifs et préventifs

Les taux de guérison signalés pour la mammite à *S. aureus* varient considérablement et semblent dépendre de nombreux facteurs spécifiques au troupeau, aux vaches ou aux souches bactériennes impliquées : les taux de transmission du troupeau, la parité des vaches, les niveaux de CCS atteints, ainsi que les caractéristiques génotypiques et phénotypiques des isolats de *S. aureus*, y compris leur niveau de production de biofilm, en sont des exemples (Barkema et al., 2006). Le traitement précoce des nouvelles infections peut être efficace; cependant, les vaches chroniquement infectées répondent généralement mal au traitement lors de la période de lactation. En fait, les traitements conventionnels approuvés atteignent rarement un taux de guérison supérieur à 20-50%, et la plupart du temps, on observe une rechute lors des lactations suivantes, démontrant que le pathogène est toujours présent ou qu'une nouvelle infection peut survenir facilement (Roy and Keefe, 2012). La persistance du pathogène dans la glande se traduit ainsi généralement en chronicité de la maladie avec des animaux voués à faire des mammites à répétition, une persistance qui peut parfois durer toute la vie de l'animal (Sutra and Poutrel, 1994). En général, les antibiothérapies prolongées ou le traitement préventif des vaches

au tarissement sont des stratégies ayant une probabilité de guérison plus élevée (Rainard et al., 2018; Roy and Keefe, 2012).

1.2.1.3 Pathogénèse de *Staphylococcus aureus* dans l'IIM

La pathogenèse de *S. aureus* lors de l'IIM chez la vache est complexe. On remarque que de multiples stratégies impliquées tout au long de la colonisation et la dissémination montrent une grande adaptation du pathogène à l'environnement hautement spécifique de la glande mammaire. Nous aborderons plusieurs éléments importants impliqués dans la virulence pouvant permettre au pathogène de résister au système immunitaire et aux traitements antibiotiques, qui aident ainsi à saisir l'extrême difficulté que représente son élimination totale de la glande mammaire.

Il existe trois phases spécifiques impliquées dans le processus d'infection à *S. aureus* : (i) l'adhésion aux cellules de l'hôte et à la matrice extracellulaire, (ii) l'invasion ou pénétration dans les tissus et (iii), l'évasion du système immunitaire de l'hôte (Middleton, 2008). De façon générale, ce processus infectieux est orchestré de façon dynamique par des régulateurs transcriptionnels permettant l'adaptation précise de l'expression des facteurs de virulence à ces différentes phases d'infection. Chez *S. aureus,* l'expression des facteurs de virulence est sous le contrôle de régulateurs globaux tels que Agr et SigB (Novick, 2003). SigB est impliqué dans la réponse à différents stress et favorise l'expression des adhésines et la formation de biofilm pour la colonisation (Horsburgh et al., 2002). En revanche, le locus Agr permet le passage de la phase de colonisation à la phase invasive selon le mécanisme du *quorum-sensing* (Novick and Geisinger, 2008). Il a été démontré que SigB est un régulateur fondamental de la virulence chez certains phénotypes de *S. aureus* particulièrement associés aux infections chroniques, notamment dans le contexte de la fibrose kystique (Mitchell et al., 2013; Tuchscherr and Löffler, 2016). Ce sujet sera abordé plus en détails dans la section 1.2.2 de ce chapitre.

Au commencement de la phase de croissance exponentielle (exponentielle précoce), des signaux activent la synthèse de la protéine A, des protéines liant la fibronectine (FnBPs) et d'autres protéines de surface permettant l'attachement initial aux tissus de l'hôte, couramment nommées MSCRAMM (*microbial surface components recognizing adhesive matrix molecules*). Lorsque la densité cellulaire bactérienne augmente, Agr diminue la production de protéines d'adhésion de surface tout en augmentant l'expression des enzymes hydrolytiques, des exotoxines et d'autres facteurs de virulence de dissémination, permettant de plus le détachement des biofilms (Otto, 2013). Ce mécanisme de *quorum-sensing* (figure 1.1) répond à la détection par AgrC de l'augmentation de la densité bactérienne par la sécrétion d'un peptide auto-inducteur (AIP; *autoinducing peptide*), résultant de l'activité coordonnée de AgrD et AgrB, qui s'accumule dans le milieu extracellulaire pour finalement atteindre une concentration critique agissant ainsi en boucle d'auto-activation sur le système à deux composants AgrC et AgrA. AgrA régule ensuite l'expression d'un ARN régulateur (ARNIII) capable de réduire significativement l'expression des protéines de surface (Novick and Geisinger, 2008). La régulation par l'ARNIII agit directement au niveau traductionnel en réprimant les adhésines ainsi que l'ARNm de *rot*, un répresseur de la synthèse des exotoxines et activateur de la synthèse des adhésines (Boisset et al., 2007). En outre, l'ARNIII permet la traduction de l'ARNm *hla* pour permettre la production de l'α-hémolysine bien connue de *S. aureus*, qui peut lyser plusieurs types de cellules hôtes.

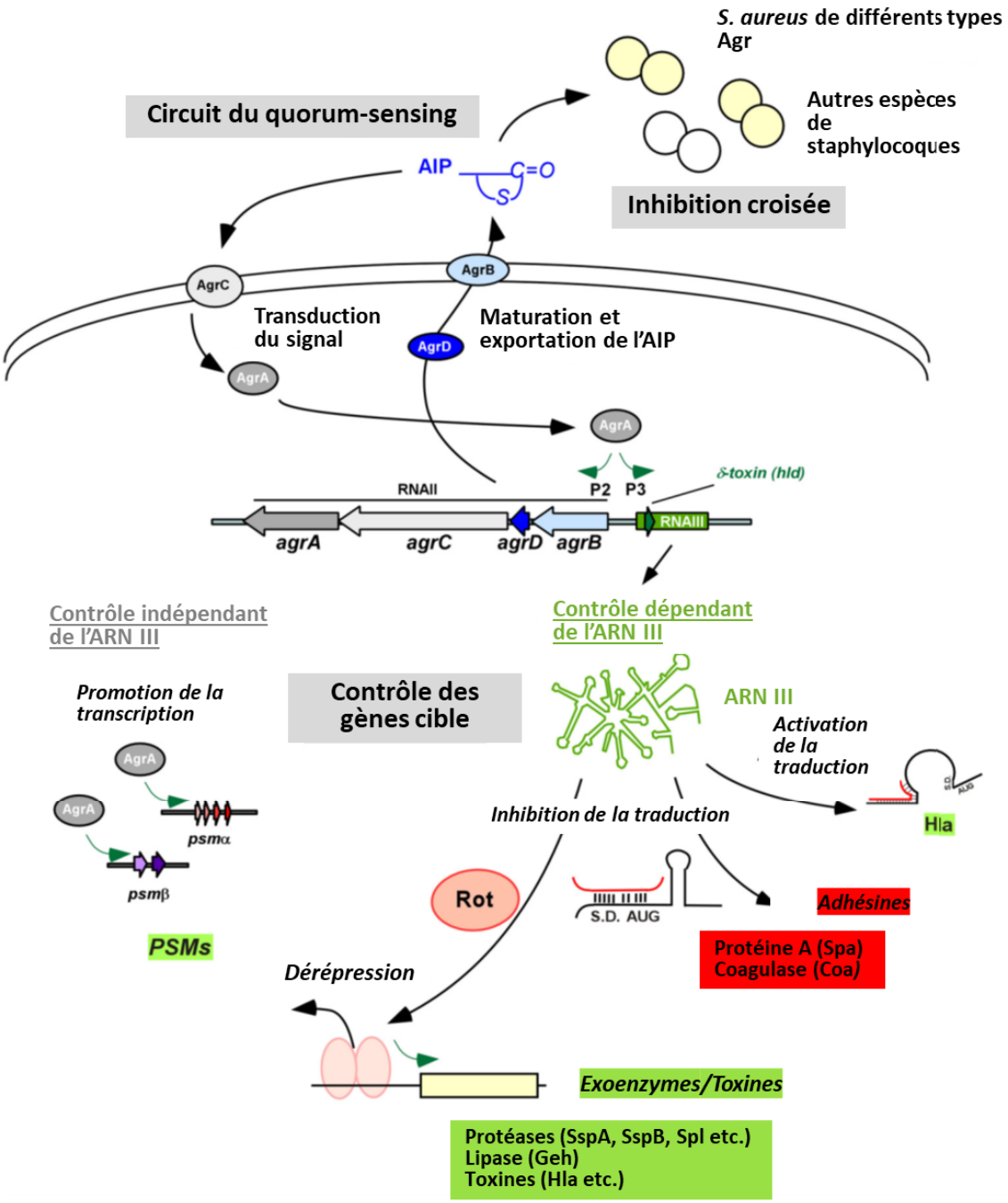

Figure 1.1. Le contrôle du système Agr chez *Staphylococcus* spp. Le circuit du *quorum-sensing* est présenté en haut à gauche. Le locus *agr* est composé de 5 gènes et produit deux transcrits distincts, RNAII et RNAIII, lesquels sont sous le contrôle de leur promoteurs

respectifs (P2 et P3). RNAII code pour AgrB, AgrD, AgrC et AgrA qui sont requis pour l'activation de la transcription au promoteur P3. Le peptide signal AIP est produit à partir du précurseur AgrD par maturation et exportation du produit par AgrB. À un certain seuil de concentration, l'AIP active le système à deux composants AgrC-AgrA, et l'AgrA phosphorylé active la transcription à partir du promoteur P2, entraînant une régulation par rétroaction positive. Une caractéristique importante d'Agr est la spécificité de type, entraînant une inhibition croisée, c'est-à-dire une inhibition de l'activité Agr par des souches appartenant à d'autres types Agr et à d'autres espèces de staphylocoques (en haut à droite). Le contrôle des gènes cible est présenté en bas. La plupart des cibles du système Agr sont régulées via l'ARNIII, dont la transcription est augmentée par AgrA, via le promoteur P3. L'ARNIII contient également le gène de la delta-toxine (*hld*). L'ARNIII contrôle les gènes cibles par appariement de bases avec les ARNm, inhibant dans la plupart des cas la traduction. Ce mécanisme est utilisé pour l'inhibition directe des gènes cibles inhibés par Agr tels que la protéine A, tandis que l'inhibition de la traduction du répresseur Rot conduit à une dé-répression de la transcription de nombreuses toxines cibles telles que l'alpha-toxine (Hla). AgrA augmente également la transcription des opérons *psm*α et *psm*β, codant pour les peptides *phenol soluble moduline* (PSM), d'une façon indépendante de l'ARNIII (adapté de Le et Otto, 2015).

Cette régulation dynamique des facteurs de virulence orchestrée par le système Agr permet d'expliquer les différentes phases d'infection dans le contexte précis de la mammite, par exemple. Lors de l'IIM, le canal du trayon (figure 1.2) représente la première barrière physique, et la porte d'entrée franchie par *S. aureus* lors de la traite, permettant ainsi la dissémination des bactéries à l'ensemble du tissus mammaire (Kerro Dego et al., 2002; Sutra et Poutrel, 1994). Après cette intrusion, la première étape dans la colonisation de la glande mammaire consiste en l'adhésion des staphylocoques aux cellules de l'hôte et à la matrice extracellulaire.

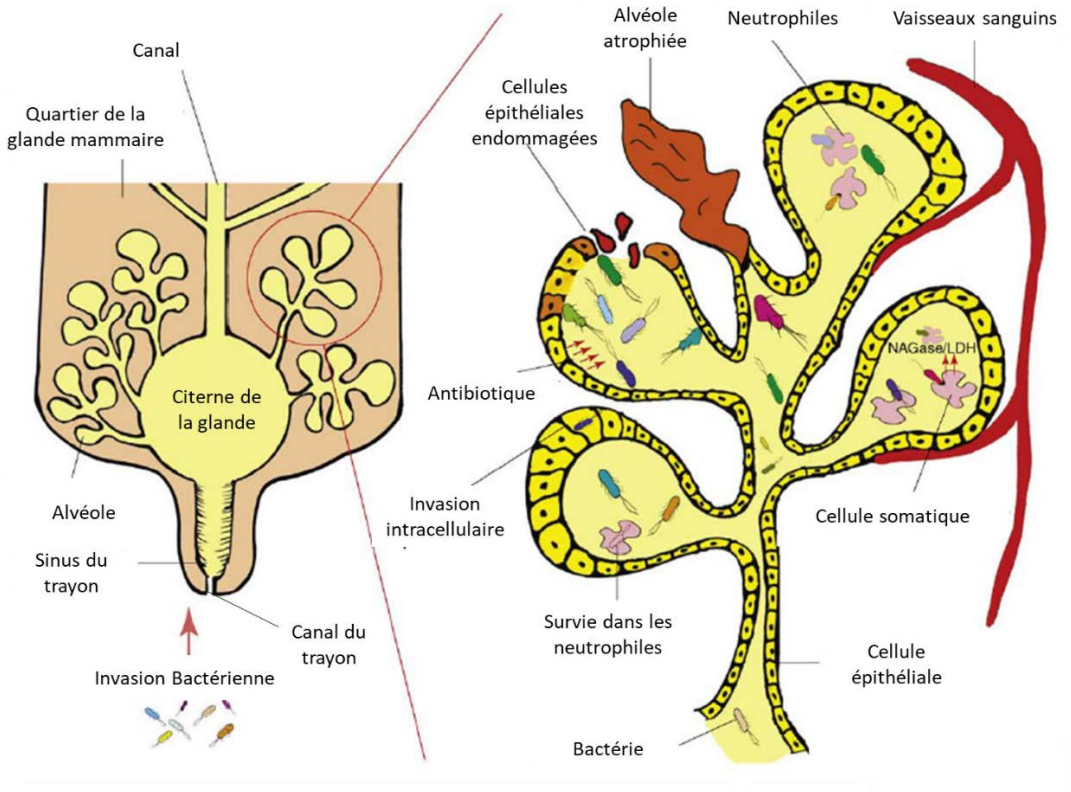

Figure 1.2. Représentation schématique du développement de la mammite dans un pis infecté. (Adapté de Viguier et al., 2009).

Cet attachement permet aux bactéries de ne pas être évacuée par le flux du lait. Les *S. aureus* expriment de nombreux facteurs de virulence impliqués dans l'adhésion, incluant notamment les protéines se liant à la fibronectine (FnBP), au fibrinogène (FgBP) et au collagène (CNA) ainsi que les clumping factor (Clf) A et B, l'acide teichoïque et certains composants de biofilms (Middleton et al., 2009; Mitchell et al., 2008). Après cette phase d'adhésion, *S. aureus* synthétise et sécrète de nombreux facteurs permettant l'invasion et la pénétration du tissu mammaire, notamment plusieurs toxines (dont les hémolysines et les leucocidines) et différentes enzymes de dégradation (protéases, coagulase, lipases, hyaluronidases) (Middleton, 2008; Suriyaphol et al., 2009). Ces lésions dans le tissu (figure 1.2) de la glande mammaire sont les principaux facteurs expliquant la réduction de production de lait, même lorsque l'infection reste inapparente (sous-clinique). Finalement, *S. aureus* produit différents facteurs lui permettant l'évasion du SI de l'hôte, d'où l'immense difficulté de succès des antibiothérapies et de la réponse immune.

Parmi ceux-ci, on peut compter les toxines superantigéniques, la protéine A, les capsules polysaccharidiques, la production de biofilms et la stratégie de persistance dans les cellules de l'hôte (figure 1.2) (Middleton, 2008).

1.2.1.4 Réponse immune de la glande mammaire à l'IIM de *S. aureus*

La glande mammaire des ruminants exploite différents types de mécanismes pour bloquer la pénétration et la dissémination des micro-organismes. Comme vu dans la section précédente sur la pathogénèse de *S. aureus*, la glande est d'abord protégée contre l'entrée d'agents pathogènes par des défenses structurelles, parmi lesquelles la barrière physique constituée par le canal du trayon est sans aucun doute l'une des plus importantes (figure 1.2). Les sphincters musculaires lisses entourant le canal du trayon empêchent également les fuites de lait en maintenant une fermeture étanche et en produisant une couche de kératine par la paroi épithéliale du conduit entre les périodes de traite (Aitken et al., 2011). Pendant la lactation, la montée en pression dans la mamelle et l'ouverture périodique de ce canal pendant la traite facilitent l'accès des bactéries au reste de la glande, où elles sont plus susceptibles de proliférer et de provoquer une infection.

La présence d'un niveau basal de ces cellules immunitaires et épithéliales résidantes dans le lait, mieux connue sous le nom de cellules somatiques, est essentielle pour surveiller et empêcher l'établissement de pathogènes dans ce milieu riche. Une fois que les bactéries sont entrées dans la citerne (figure 1.2), l'immunité innée puis adaptative de l'hôte prennent le relais et fonctionnent de manière coordonnée. Ceci est amorcé par l'activation des voies des récepteur de reconnaissance de motifs moléculaires (PRRs) et la production subséquente de cytokines pro-inflammatoires (TNF-α et l'IL-1β, entre autres) (Oviedo-Boyso et al., 2007). Ces cytokines spécifiques favorisent le recrutement rapide de spécialistes cellulaires, tels que les neutrophiles et les macrophages, migrant de façon plus importante dans la glande depuis la périphérie pour ingérer directement des microorganismes par phagocytose et produire des molécules de défense solubles diverses (Aitken et al., 2011; Ezzat Alnakip et al., 2014). Les neutrophiles sont les cellules phagocytaires les plus importantes dans la glande mammaire, et constituent la défense

cellulaire dominante contre les agents pathogènes causant la mammite (Rainard et Riollet, 2006), en particulier pour *S. aureus* (Spaan et al., 2013).

Il a été établi que le type de pathogène influence grandement la réponse de l'hôte bovin à l'infection (Petzl et al., 2018). La plupart des espèces de coliformes qui causent la mammite provoquent une réponse inflammatoire aiguë marquée par rapport à l'IIM de *S. aureus* en raison de la présence du lipopolysaccharide (Ezzat Alnakip et al., 2014). En effet, l'issue d'une mammite clinique causée par des coliformes est complètement différente de celle d'une IIM sous-clinique (Jensen et al., 2013), qui est caractérisée par une suppression marquée de la réponse immune (Günther et al., 2017). Cette immunomodulation, ainsi que la capacité de *S. aureus* d'entrer et de persister dans les cellules hôtes lui permettrait donc d'établir des infections sous-cliniques devenant chroniques.

1.2.2 Le phénotype *small-colony variant* (SCV) de *S. aureus*

Conjointement, d'autres aspects particuliers de la pathogénèse de *S. aureus* sont à considérer en ce qui a trait à sa chronicité. Lors des dernières décennies, une sous-population phénotypique de *S. aureus* spécialement associée à la persistance chez l'hôte a été isolée et caractérisée dans de nombreux cas de maladies chroniques ; les *small-colony variants* (SCVs). Comme l'indique l'appellation, le principal attribut de ces isolats consistait en une croissance plus lente menant à la formation de petites colonies d'environ le dixième de la taille normale. Ce phénotype a aussi pu être observé chez des espèces bactériennes aussi diverses que *S. epidermidis*, *S. capitis*, certains sérovars de *Salmonella*, *P. aeruginosa*, *E. coli*, *V. cholerae*, *N. gonorrhoeae*, *S. marcescens* ou *L. acidophilus*.

1.2.2.1 Caractéristiques phénotypiques et bases génétiques

Chez *S. aureus*, les traits phénotypiques communs associés aux SCVs sont la formation de petites colonies non pigmentées et non hémolytiques sur milieu de culture solide. Ces

caractéristiques peuvent être expliquées par le profil métabolique particulier des SCVs. On considère que la majorité des SCVs cliniques de *S. aureus* découleraient de mutations précises amenant des déficiences dans la chaîne de transport des électrons, altérant le processus de phosphorylation oxydative.

Brièvement, chez une souche de *S. aureus* au phénotype normal, le NADH et le $FADH_2$ produits lors du cycle de Krebs sont utilisés pour générer des quantités élevées d'ATP via leur prise en charge par les complexes membranaires de la chaîne de transport au gradient de potentiel réducteur croissant. Le mouvement d'électron de part et d'autre de la membrane est accompagné de l'accumulation de protons (H^+) à l'extérieur de la membrane bactérienne qui est utilisée ultimement par l'ATP synthase. Le phénotype de ce type de SCV serait ainsi expliqué par des altérations rendant ces complexes inactifs, affectant le métabolisme.

En effet, les SCVs les plus étudiés à ce jour sont incapables de synthétiser le ménadione ou l'hémine, deux molécules oxydables associées aux complexes de la chaîne de transport des électrons. Des mutations spécifiques dans les voies de synthèse de celles-ci ont été observées chez ces dernières, notamment dans les gènes *menD* et *hemB*, respectivement. Un second type de SCV a également été caractérisé, le thymidine-dépendant, et démontre un phénotype en tout point semblable à ceux qui sont déficients dans la chaîne de transport des électrons, mais ne peut pas survivre du tout sans la présence de thymidine (Proctor et al., 2006).

Les SCVs sont en outre reconnus pour une capacité accrue à résister à l'action des molécules antimicrobiennes. Plusieurs études ont déjà fait état de leur résistance alarmante à de nombreux antibiotiques (Gläser et al., 2014; Vaudaux et al., 2011), notamment aux aminoglycosides, qui sont dépendants du potentiel membranaires ($\Delta\Psi$) pour leur mode d'action (Proctor et al., 2006).

1.2.2.2 Prévalence dans les infections

Chez *S. aureus*, les SCVs sont fréquemment isolés lors d'infections chroniques de l'homme et des animaux. Depuis quelques décennies, on l'a isolé d'échantillons cliniques de certains cas d'infections particulièrement difficiles à traiter. Parmi ceux-ci, on peut citer des infections des voies respiratoires de patients atteints de fibrose-kystique, infections profondes post-chirurgie, ostéomyélites chroniques ou encore des infections de corps étrangers tels que les prothèses ou implants articulaires (Kalinka et al., 2014; Proctor et al., 2006). Malheureusement, il semblerait que la véritable prévalence des SCVs dans les infections soit encore grandement sous-évaluée, pouvant ultimement mener à des échecs thérapeutiques (Yagci et al., 2013); d'où l'intérêt grandissant pour le développement de méthodes d'identification et d'isolement de ce phénotype. Les SCVs ont déjà été retrouvés chez le bovin laitier (Alkasir et al., 2013; Atalla et al., 2008, 2011), mais tel que discuté précédemment, leur présence est grandement sous-évaluée car elle se retrouve souvent sous la forme de populations mixtes, et leur croissance est facilement masquée ou surpassée par les bactéries du phénotype normal.

1.2.2.3 Facteurs d'émergence et caractéristiques transcriptionnelles favorisant la persistance

On considère ainsi que certains facteurs environnementaux favoriseraient l'émergence du phénotype, notamment l'utilisation de traitements antibiotiques. Par exemple, le SCV dépendant à la thymidine a été particulièrement souvent isolé de poumons de patients atteints de fibrose kystique traités à long terme au sulphamethoxazole (SMX) (Proctor et al., 2006). Également, il a été depuis longtemps observé que le milieu intracellulaire contribuait à favoriser l'émergence des SCVs (Vesga et al., 1996).

De plus, plusieurs facteurs transcriptionnels donnent au phénotype SCV des caractéristiques particulières lui permettant la persistance chez l'hôte. On a souvent cité leur capacité augmentée à la production de biofilm (Mitchell et al., 2010) et à une internalisation et survie accrue dans

les cellules de l'hôte (Brouillette et al., 2004; Kalinka et al., 2014). Comme nous l'avons vu en 1.2.1.3, l'expression des facteurs de virulence de *S. aureus* est sous le contrôle des régulateurs globaux Agr et du facteur alternatif SigB. Rappelons que le locus Agr régule à la hausse les facteurs de virulence invasifs lors d'une grande densité bactérienne. En contrepartie, le facteur de transcription alternatif SigB est impliqué dans la réponse à différents stress et favorise plutôt l'expression d'adhésines pour la colonisation (Mitchell et al., 2008). Chez les SCVs, l'expression des facteurs de virulence est influencée préférentiellement par SigB plutôt que par Agr, qui n'est plus activé à la fin de la phase exponentielle comme dans les souches à phénotype régulier (figure 1.3). Lors d'une pression sélective (par exemple, un traitement antibiotique, la réponse immunitaire de l'hôte ou le milieu intracellulaire), le phénotype SCV peut émerger, et suite à libération de cette pression, on peut assister à la perte de la mutation et à la réémergence du phénotype «agressif», hautement favorisé par ses performances de croissance et de dissémination (figure 1.3).

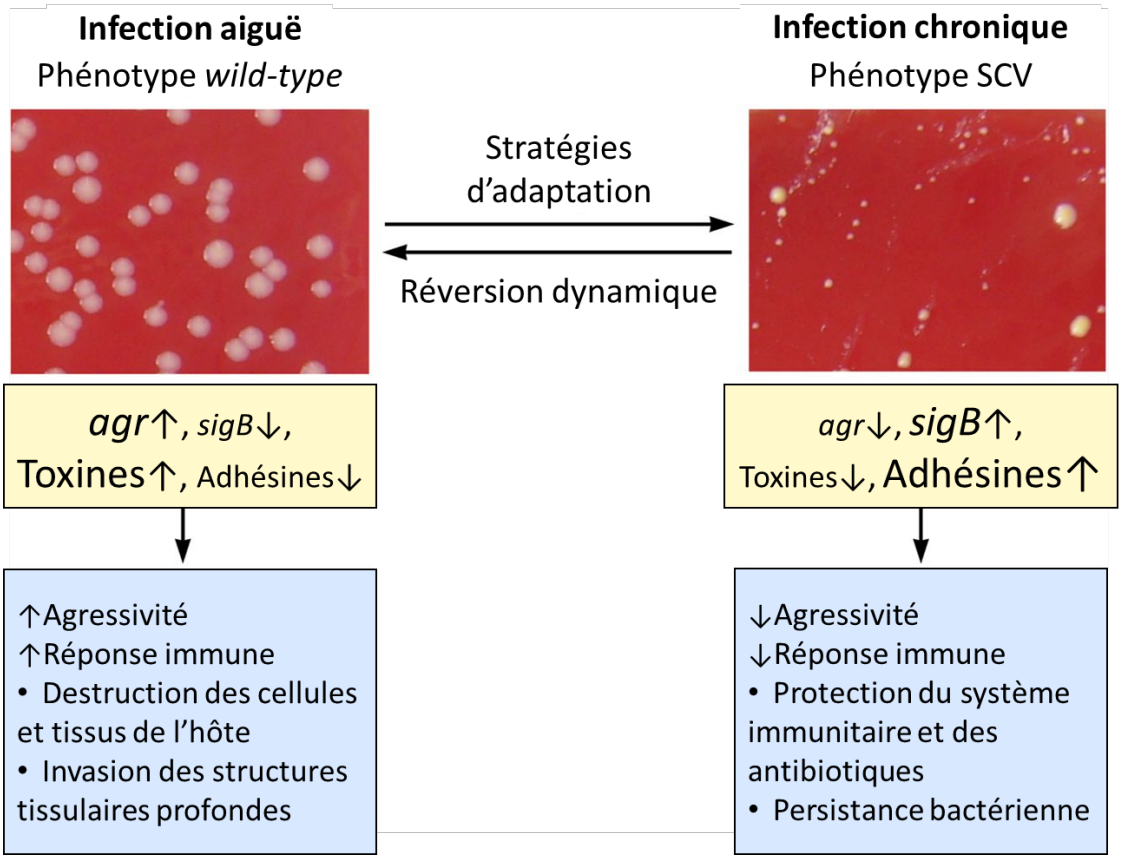

Figure 1.3. Développement dynamique des SCVs au cours de l'évolution de l'infection aiguë à chronique. Lors d'une infection aiguë, les bactéries présentent un haut niveau d'expression d'*agr* et des toxines régulées par celui-ci, provoquant des réactions inflammatoires et cytotoxiques. Au cours de l'évolution chronique de l'infection, les bactéries appliquent des mécanismes d'adaptation et de résistance qui impliquent la formation dynamique de phénotypes de type SCV, une régulation négative de l'opéron *agr* et une régulation positive de SigB. En quittant par exemple le milieu intracellulaire, les SCVs peuvent rapidement revenir au phénotype normal, ou *wild-type*, complètement agressif, ce qui peut provoquer un nouvel épisode d'infection (adapté de Kahl et al., 2016).

On sait maintenant que cette réversion phénotypique est un processus hautement dynamique, inhérent à la pathogénèse de *S. aureus* (Edwards, 2012) et ainsi pourrait permettre facilement l'établissement d'infections chroniques (Tuchscherr et al., 2011). Il est généralement admis que

la survie prolongée des SCVs est attribuable au moins en partie à une expression diminuée d'hémolysine-α, tandis que leur plus grande capacité d'invasion est due à une plus grande expression des FnBPs (Tuchscherr et al., 2010; Vaudaux et al., 2002). Ainsi, la persistance de staphylocoques intracellulaires pourrait contribuer à l'établissement d'infections chroniques en permettant à la bactérie d'échapper à l'action des antibiothérapies et du SI (Brouillette et al., 2004; DeLeo and Otto, 2008).

1.2.2.4 Construction de SCVs génétiquement stables

Les SCVs cliniques sont relativement difficiles à étudier, notamment en raison du phénotype souvent instable, prompt à la réversion au phénotype normal dans les conditions de laboratoire. Afin de mieux les caractériser, des SCVs génétiquement définis notamment via l'interruption du gène *hemB*, ont été obtenus dans différentes études. Cette stratégie avait été utilisée notamment pour tester l'hypothèse que cette déficience promouvait la persistance intracellulaire (Von Eiff et al., 1997) puisque les SCVs avaient démontré être la cause de cas cliniques d'infections récurrentes qui réapparaissaient plusieurs années après le traitement de l'infection initiale. Chez les SCVs au gène *hemB* inactivé, on observe en effet l'accumulation de NAD(P)H et $FADH_2$, un potentiel membranaire réduit et des concentrations d'ATP basses (Senn et al., 2005). Plusieurs gènes impliqués dans la glycolyse et la fermentation sont régulés à la hausse, alors que les enzymes des acides tricarboniques sont régulés à la baisse, démontrant l'altération dans le métabolisme menant à une production d'énergie moindre (Kohler et al., 2003).

1.2.3 La résistance aux peptides cationiques antimicrobiens chez *S. aureus*

Les peptides cationiques antimicrobiens (CAMPs) représentent la première ligne de défenses immunitaires innées sur la peau des mammifères et font également partie des mécanismes par lesquels les bactéries sont éliminées dans le phagolysosome des neutrophiles suite à la phagocytose (Joo and Otto, 2015). De nombreux organismes différents (des procaryotes à l'humain) produisent ces peptides, et nombre de ces molécules ayant été reconnues comme

atives contre les staphylocoques ont été découvertes chez l'homme ou les animaux. La majorité sont typiquement composés de sequences peptidiques amphipathiques avec une charge positive nette à pH physiologique, et partagent des structures secondaires d'hélices-α ou feuillet-β bien définies (Pietiäinen et al., 2009). En raison de leurs propriétés cationiques, les CAMPs peuvent facilement se lier aux membranes bactériennes, fortement chargée négativement, et sont ainsi capables de s'intégrer dans les membranes cellulaires et d'y former des pores menant ainsi à la lyse cellulaire (Sahl et al., 2005).

En contre-partie, les bactéries ont développé un certain nombre de mécanismes pour se défendre contre l'activité des CAMPs. Ces mécanismes de résistance comprennent entre autres : diminuer leur affinité pour les CAMPs (ou augmenter leur répulsion) en substituant des constituants de surface cellulaire anioniques par des molécules cationiques; la biosynthèse augmentée de la paroi cellulaire ou la synthèse de protections polysaccaridiques (ex : capsule, biofilm); des mécanismes de piégeage externes qui séquestrent ou neutralisent les CAMPs par sécrétion directe de protéines; l'exportation par des pompes à efflux; et la production de peptidases pour l'inactivation et la dégradation. Des exemples des systèmes utilisés en particulier chez *S. aureus* pour chacun de ces mécanismes sont illustrés à la figure 1.4.

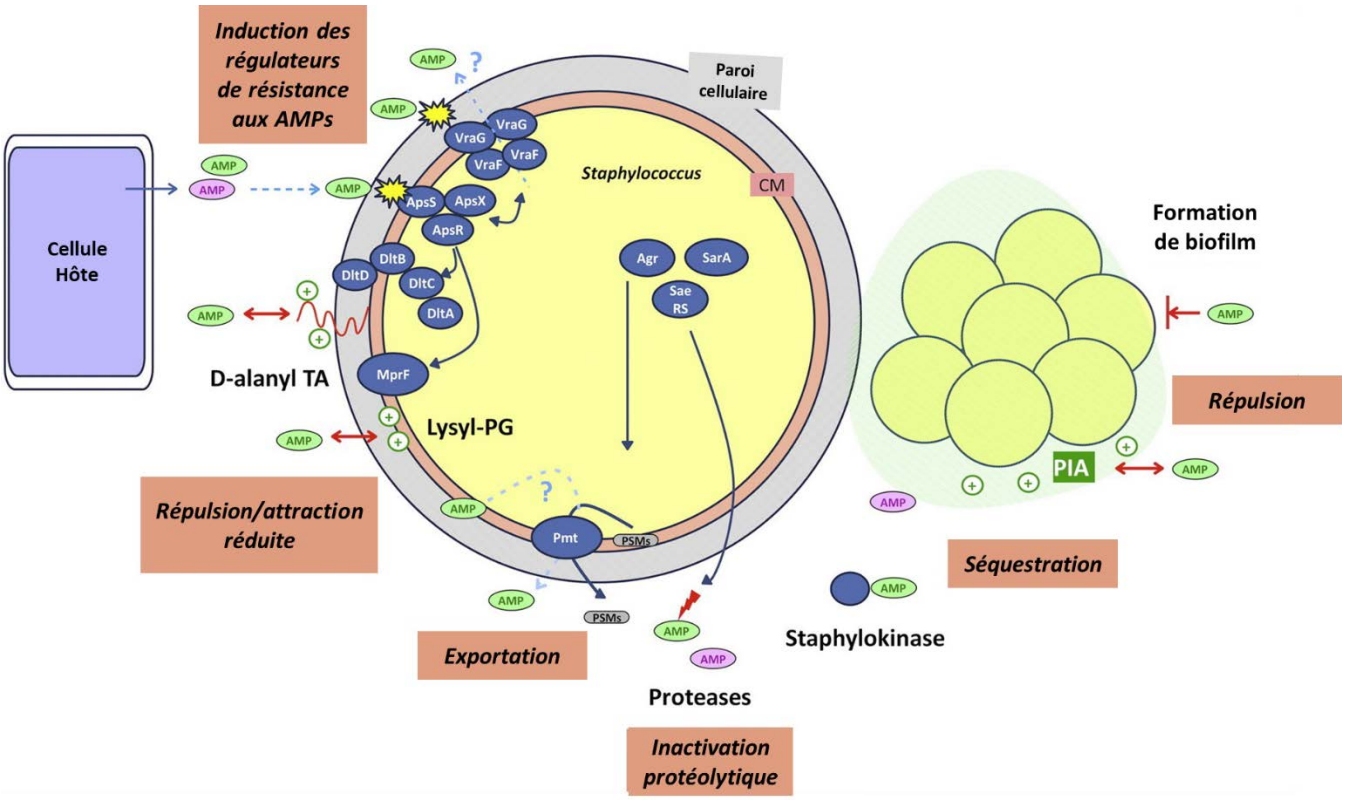

Figure 1.4. Mécanismes de résistance aux CAMPs chez les staphylocoques. Les cellules hôtes produisent des CAMPs (chargés positivement, en vert) ou, rarement, des AMPs anioniques, chargés négativement tels que la dermcidine (en rouge). Les CAMPs induisent le système de régulation ApsXRS-VraFG (ou GraXRS-VraFG chez *S. aureus*) conduisant à une expression accrue du système Dlt, qui permet l'ajout de résidus D-alanine sur les acides téichoïques de la paroi bactérienne, et MprF, qui modifie le phosphatidylglycerol avec des résidus lysyl. Les deux mécanismes contribuent à la répulsion/attraction réduite des CAMPs. Des pompes d'exportation telles que le transporteur Pmt expulsent de la membrane cellulaire bactérienne les molécules toxiques telles que les *phenol-soluble modulins* (PSM) staphylococciques, mais aussi éventuellement les CAMPs produits de l'hôte. Les protéases sécrétées avec une faible spécificité de substrat dégradent les peptides antimicrobiens (AMPs) (à la fois les AMPs anioniques et cationiques) et sont régulées par des régulateurs globaux tels que Agr, SarA et SaeRS. La staphylokinase inactive les CAMPs par séquestration. La formation

de biofilms contribue à la résistance aux peptides antimicrobiens par une multitude de mécanismes qui incluent une diminution de leur pénétration. De plus, le polysaccharide associé aux biofilms PIA (ou PNAG) possède une charge nette positive et a ainsi la capacité de repousser/séquestrer les CAMPs (adapté de Joo et Otto, 2015).

Chez *S. aureus*, le système de détection/résistance aux CAMPs Aps se nomme GraXRS-VraFG (figure 1.5). GraS et GraR correspondent respectivement aux composantes senseur et régulateur de réponse d'un système de détection à deux composantes classique, tandis que le rôle précis de la protéine accessoire GraX n'est pas encore élucidé. L'opéron *vraFG* code pour un module ayant une homologie de séquence très proche des systèmes de type ABC transporteur composés d'une ATP *binding cassette* et d'une perméase. Il convient de noter que VraFG ne fonctionne pas comme un module de détoxification comme on l'avait auparavant cru (Meehl et al., 2007), car il ne confère pas de résistance accrue lorsque produit ou surexprimé seul (Falord et al., 2012). Il a plutôt été démontré que tout en étant sous la régulation directe du système à deux composants GraXRS, VraFG joue également un rôle essentiel dans la détection de la présence des CAMPs et la signalisation via GraS pour activer la transcription dépendante de GraR, y compris sa propre transcription, tel qu'illustré à la figure 1.5 (Falord et al., 2012). L'expression de deux facteurs déterminants, *mprF* et *dlt*, sont dépendants de *graXRS-vraFG* (figure 1.5), et sont les effecteurs directement responsables de la résistance par la modification de la charge nette de la surface bactérienne (Meehl et al., 2007). En effet, *mprF* code pour une lysyl-phosphatidylglycérol synthase qui ajoute des molécules de lysine chargées positivement au phosphatidylglycérol dans la membrane cellulaire, tandis que l'opéron *dltABCD* contribue également à la charge nette positive en attachant de manière covalente des résidus D-alanine aux acides téichoïques de la paroi cellulaire. Lorsque qu'un ou des éléments de ce système sont altérés, une susceptibilité augmentée aux peptides cationiques est observée (Yang et al., 2012).

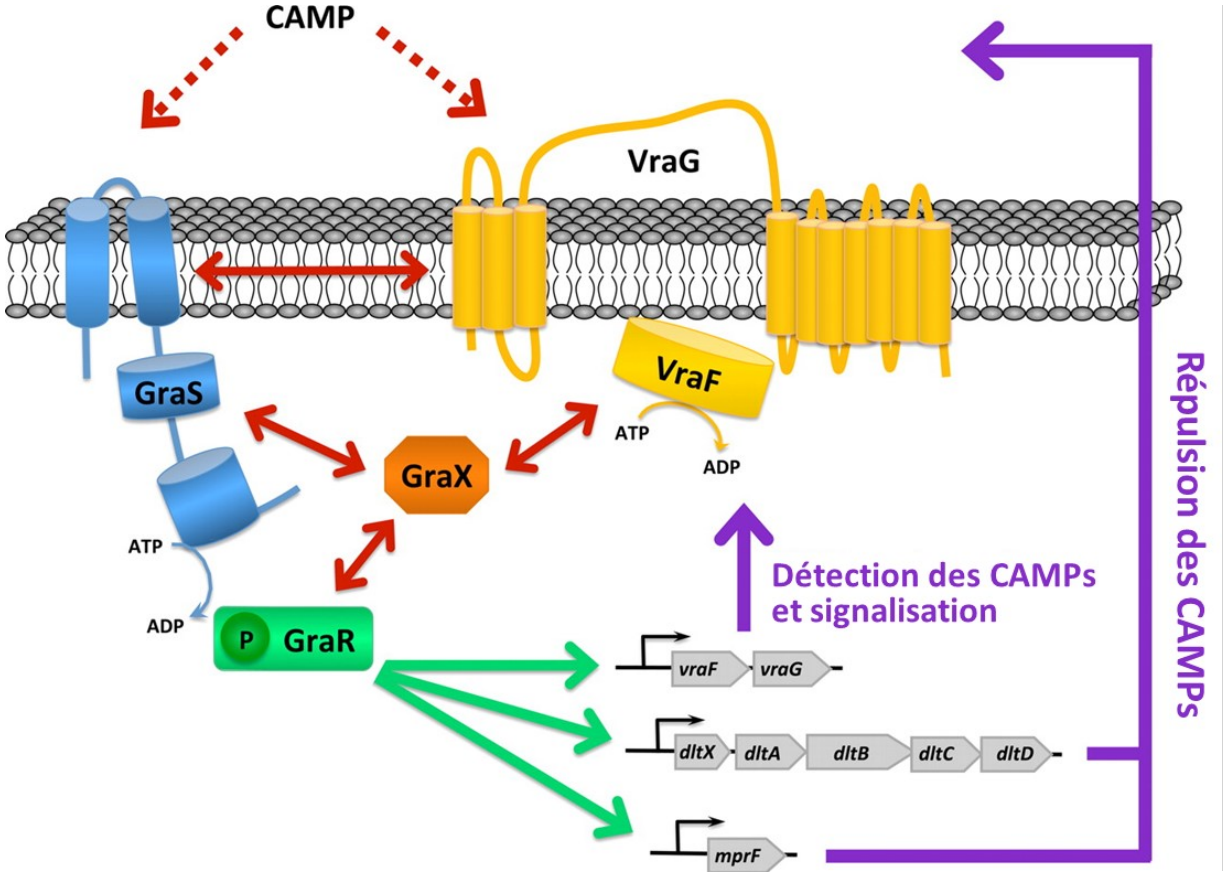

Figure 1.5. Le système de détection GraXRS-VraFG. Ce système de détection à 5 composantes contrôle la détection et la résistance aux CAMPs chez *S. aureus*. Les CAMPs sont détectés par GraS mais aussi VraFG, probablement à travers la boucle extracellulaire de la perméase VraG, et le signal est transduit vers GraS via un mécanisme qui implique une interaction entre VraG et GraS. L'activation du système GraSR conduit à une transcription accrue de l'opéron *dlt* et du gène *mprF*, conduisant à une résistance accrue au CAMPs. Les flèches à deux pointes indiquent les interactions protéine-protéine, les flèches vertes indiquent l'activation de la transcription et les flèches violettes indiquent la synthèse des protéines et le phénotype engendré (adapté de Falord et al., 2012).

1.3 Stratégies de vaccination contre les IIMs à *S. aureus*

1.3.1 Avantages de la vaccination sur l'antibiothérapie

Tel que discuté précédemment, les options efficaces pour combattre les IIMs à *Staphylococcus aureus* sont rares pour les acteurs de l'industrie laitière, et pour le moment ne consistent qu'au traitement antibiotique des vaches lorsque le pathogène est isolé des quartiers, ou que des signes cliniques apparaissent. Cette stratégie est d'une part inefficace, car les vaches présentant une infection déjà bien établie réagissent mal au traitement durant la période de lactation, comme nous l'avons vu dans la section 1.2.1.2. La persistance du pathogène dans la glande se traduit généralement en cas de mammites chroniques qui deviennent de plus en plus difficiles à traiter à long terme.

D'autre part, cette stratégie ne couvre pas l'ampleur du problème occasionné par les infections sous-cliniques, qui sont invisibles au niveau de l'état général de l'animal. En effet, tel que discuté précédemment, *S. aureus* est majoritairement responsable de mammites sous-cliniques (Sutra et Poutrel, 1994) et donc passe régulièrement sous le radar des producteurs. Le traitement préventif et systématique de toutes les vaches est impossible dans le contexte de l'industrie laitière. Contrairement à l'élevage porcin, par exemple, l'utilisation d'antibiotiques pour traiter les bovins laitiers d'élevage est très réglementée et surveillée. Toute utilisation pendant la lactation est soumise à une période de retrait du lait, et ce même si l'injection ne se fait pas au niveau de la glande mammaire. De surcroit, ce retrait équivaut à de nouvelles pertes monétaires substantielles pour l'éleveur. Cette présence constante et inaperçue au sein du troupeau, couplée aux difficultés d'élimination de la glande à long terme, contribuent à maintenir un réservoir d'infection dans les troupeaux et permet la transmission contagieuse du pathogène de vache en vache lors de la traite (Olde Riekerink et al., 2008).

Finalement, il est important de noter qu'il existe des risques associés à l'antibiothérapie, et l'utilisation systématique d'antibiotiques est de plus en plus contestée car elle stimule à la

longue le développement de résistances. La propagation des gènes de résistance aux antibiotiques par transfert horizontal (Li et Zhao, 2018) et l'émergence de souches de *S. aureus* résistants à la méticilline (MRSA) associées aux animaux, mais pouvant être transmises aux humains, sont particulièrement préoccupantes (García-Álvarez et al., 2011; Holmes and Zadoks, 2011). Ainsi, la pression du public pour réduire l'utilisation d'antibiotiques dans la production animale est une incitation importante à trouver des mesures efficaces de lutte non antibiotique contre cette maladie.

Un vaccin dirigé contre *S. aureus* constituerait donc un outil de choix pour la prévention et le traitement des IIMs à *S. aureus* : la vaccination permettrait à la fois de réduire l'incidence des nouveaux cas d'IIM, et donc la présence du pathogène dans les troupeaux, et de réduire les manifestations sous-cliniques (baisse de la production et de la qualité du lait) ou cliniques chez des vaches infectées. De plus, le processus d'homologation pour un vaccin est considéré beaucoup plus court et moins couteux que pour un antibiotique, ce qui laisse supposer qu'un vaccin serait mis sur le marché plus rapidement qu'un antibiotique

Le développement d'un vaccin visant la prévention et le contrôle des IIMs à *S. aureus* est donc d'un intérêt considérable pour l'industrie laitière, et un sujet d'actualité à la source de plusieurs études publiées lors des dernières années. Malheureusement, la polyvalence du pathogène à exprimer différents facteurs de virulence dans la glande, ses nombreuses méthodes de camouflage du SI et la variabilité phénotypique des souches pouvant causer la maladie ont mené à peu de succès lors des dernières décennies. En effet, on considère qu'aucune formulation classique n'a pu démontrer de grande efficacité de protection à ce jour (Rainard et al., 2018). Pourtant, l'amélioration des technologies immunologiques et du génie génétiques ainsi qu'une meilleure compréhension du fonctionnement de l'immunité dans les infections à *S. aureus* semblent ouvrir la porte à de nouveaux progrès.

1.3.2 Objectifs visés par la vaccination

Les buts spécifiques de la vaccination contre la mammite sont multiples et peuvent consister en : (i) la diminution de la sévérité des symptômes cliniques, (ii) la réduction des CCS dans le lait ou (iii) en la réduction de l'incidence de nouveaux cas de mammites dans un troupeau. Le type de mammite et le pathogène responsable de celle-ci peut faire varier l'objectif de vaccination. Par exemple, la vaccination contre le pathogène environnemental et non contagieux *E. coli* peut viser la réduction des symptômes cliniques, qui sont en général plus sérieux et aigus avec les coliformes (Cebra et al., 1996). À titre d'exemple, le vaccin injectable et commercialisé J5 est efficace pour la réduction des symptômes cliniques des mammites à *E. coli* (Hogan et al., 1995), mais d'une faible efficacité au niveau préventif et protecteur à grande échelle (Wilson et al., 2007).

En contraste, la gravité des infections à *S. aureus* réside principalement dans le fait qu'elles sont à l'origine d'importantes pertes économiques dues à la diminution progressive de la production et de la qualité du lait (Aghamohammadi et al., 2018), plutôt que de l'effet direct de symptômes cliniques ou de mortalité des animaux. Ainsi, sur la plupart des fermes, le but visé par la vaccination contre les mammites à *S. aureus* réside dans la prévention des nouvelles IIMs, puisque les formes cliniques aigues de la maladie sont rares, alors que la dissémination contagieuse est commune (Middleton, 2008).

1.3.3 Cribler et cibler les antigènes exprimés pendant l'infection

Trouver le ou les antigènes qui permettront de protéger contre un pathogène tel que *S. aureus* est ardu (**Annexe I**). Comme mentionné précédemment, les souches de *S. aureus* responsables de la mammite bovine sont diverses et peuvent être phénotypiquement très différentes. Par conséquent, un antigène protecteur commun qui serait utile contre plusieurs souches n'a pour l'instant pas encore été découvert. De plus, l'expression des antigènes de *S. aureus*, à la pathogénèse complexe, peut être modulée entre les conditions *in vitro* et *in vivo*, et varie

considérablement selon la phase de l'infection et en réponse aux environnements extracellulaires et intracellulaires (Allard et al., 2006; Lowe et al., 1998). Il est donc important de trouver des stratégies de criblage antigénique mettant à profit les avancées biotechnologiques récentes et prenant en compte cette disparité.

Des études se sont déjà penchées sur la recherche de protéines et de gènes qui pourraient être exprimés *in vivo* en utilisant des conditions de culture *in vitro* qui tentaient d'imiter l'environnement de la glande mammaire et du lait (Lammers et al., 2000; Taverna et al., 2007). Parce que ces milieux de croissance alternatifs peuvent difficilement reproduire l'environnement complexe de la glande mammaire, une approche intéressante utilisée par notre équipe de recherche a été de recueillir le lait de vaches expérimentalement infectées à *S. aureus* pour examiner, par une approche transcriptomique, les gènes qui étaient fortement exprimé par pendant l'IIM par plusieurs souches et chez plusieurs vaches de façon soutenue dans le temps (Allard et al., 2013). Cette approche a conduit à l'identification de gènes et de protéines candidates pour le développement de vaccins ou de médicaments qui seront abordés plus loin (section 1.5).

Dans une étude récente, une approche immunoprotéomique a été utilisée pour identifier des protéines antigéniques de la surface de *S. aureus* pour trouver de nouveaux candidats de vaccin potentiels (Misra et al., 2018). Plus spécifiquement, les protéines exprimées par des bactéries cultivées dans des conditions restreintes en fer pour imiter l'environnement de l'hôte ont été extraites, séparées et détectées par immunobuvardage spécifique avec des anticorps du lait de mammite. Trente-huit protéines spécifiques de *S. aureus* ont pu être détectées par des anticorps présents chez plusieurs vaches, dont 8 auraient été associées à la surface et préalablement déterminées comme étant impliquées dans la virulence du pathogène.

1.3.4 Formulations vaccinales et cibles antigéniques mises à l'essai

Parmi les vaccins contre les IIMs à *S. aureus* traditionnellement mis à l'essai, on compte plusieurs types de formulations : des suspensions de bactéries tuées ou complètement lysées, des extraits de parois cellulaires issus de souches de laboratoire ou de terrain sélectionnées. Ces techniques de vaccination existent depuis des décennies, et sont relativement simples et peu coûteuses à produire. L'essor des nouvelles techniques immunologiques et biomoléculaires des dernières années a permis d'utiliser de nouvelles stratégies pour éliciter des réponses beaucoup plus ciblées contre certains composantes précis de *S. aureus*. Malheureusement, malgré un certain succès à éliciter des réponses fortes et spécifiques, la plupart finissent par être délaissées en raison de leur efficacité de protection limitée lors d'essais à grande échelle chez la vache (Leitner et al., 2011). Cet important aspect de protection difficile à obtenir explique la rareté des nouvelles technologies réussissant à compléter la traversée du processus d'homologation. Les différentes formulations de vaccination et quelques études représentatives du niveau de protection de ces candidats vaccins seront discutés ici en détail.

1.3.4.1 Vaccins inactivés et lysats cellulaires

Ce mode de vaccination très simple consiste en l'utilisation de microorganismes entiers (inactivés/tués; en général par la chaleur) ou de lysats produits à partir de ces derniers associés ou non à des adjuvants. Les vaccins tués (ou bactérines) ne sont généralement pas capables d'induire de réponse immune à méditation cellulaire (en particulier la réponse CTL) et de plus nécessitent l'ajout d'un adjuvant afin d'augmenter leur pouvoir protecteur. Ils sont cependant très efficaces pour induire une forte production d'anticorps capables de reconnaître les épitopes de surface du pathogène.

En médecine vétérinaire, l'utilisation de ce type de vaccins a été abondamment étudiée depuis plusieurs années, et le premier vaccin commercialisé contre les mammites à *S. aureus* a été

utilisé aux États-Unis à partir des années 1970 (Lysigin®, Boehringer Ingelheim, Vetmedica, Inc). Il est composé de lysats issus de 5 souches de *S. aureus* exprimant les 3 types de capsules polysaccharidiques prédominants lors de mammites bovines (5, 8 et 336). De nombreuses études ont évalué ce vaccin, démontrant la réduction des symptômes cliniques ainsi qu'une augmentation des cas de guérison spontanée (Middleton et al., 2009). Cependant, aucune différence n'a été observée tant au niveau des CCS individuels dans le lait que dans l'élimination bactérienne de la glande. Au niveau de la réponse élicitée, une quantité plus élevée d'IgGs totaux contre *S. aureus* a également été démontrée dans le groupe vacciné, (Luby et al., 2007), mais le nombre d'anticorps ospsonisants présents dans le lait pourrait ne pas être suffisant pour permettre la protection (Middleton et al., 2009).

Plus récemment, l'utilisation de souches productrices de biofilms sous la forme inactivée a permis de réduire de manière intéressante la charge bactérienne des glandes mammaires de génisses infectées expérimentalement (Pérez et al., 2009). Cette réduction serait liée notamment à l'induction d'anticorps spécifiques dirigés contre certains composés polysaccharidiques présents dans les biofilms, dont la Poly-N-acetylglucosamine (PNAG) et le *slime associated antigenic complex* (SAAC). L'ensemble de ces travaux ont abouti à la commercialisation du vaccin Startvac®-TopVac® (Hipra, Inc., Espagne) en Europe et en Amérique du nord. Celui-ci est composé d'une souche de *S. aureus* pouvant exprimer le SAAC et de la souche d'*E. coli* J5, tous deux inactivés et associés à un adjuvant dans une émulsion d'huile. Bien que l'efficacité du vaccin à grande échelle ne soit pas encore établie, une récente étude a pu démontrer un certain effet positif de la vaccination contre les nouvelles mammites à *S. aureus* et aux staphylocoques à coagulase négative (CNS) sur deux fermes (Schukken et al., 2014). L'efficacité du vaccin a été estimée en se basant sur le taux de transmission de nouvelles infections et les paramètres de durée de celles-ci, menant au final à une réduction modérée de l'incidence de nouvelles IIMs aux staphylocoques et une réduction plus marquée de la durée totale des IIMs. Cependant, des divergences significatives dans cette efficacité ont été détectées entre les deux fermes, un fait pouvant éventuellement être associé à des pratiques de gestion différentes, qui sont connues pour affecter la dynamique d'infection, et pourraient donc avoir grandement contribué à

l'efficacité globale observée (Scali et al., 2015). Le bénéfice mitigé de ce vaccin pourrait aussi être attribué à la diversité des souches de *S. aureus*, qui varie géographiquement, leur degré de production de SAAC et de biofilm qui peut dépendre de la phase de croissance bactérienne ou de l'environnement (Bradley et al., 2015; Landin et al., 2015; Schukken et al., 2014).

Les vaccins inactivés ne semblent donc pas encore avoir fait leurs preuves, pour deux raisons généralement évoquées: 1) leur manque de spécificité antigénique pouvant avoir l'effet de « diluer » la réponse immune protectrice; 2) le problème de la différence phénotypique des souches pouvant causer la mammite rendant leur utilisation parfois questionnable. D'autres stratégies visant la conception d'un vaccin contenant des antigènes clés, définis, et à large spectre d'activité sont donc aussi envisagées, bien qu'elles comportent également certains défis. À cet égard, plusieurs tentatives pour développer plutôt une réponse contre des composantes précis de *S. aureus* ont été effectuées via l'utilisation des vaccins sous-unitaires.

1.3.4.2 Vaccins sous-unitaires protéiques

L'utilisation de vaccins contenant des organismes pathogènes entiers décrits précédemment réduit le besoin d'identifier les antigènes protecteurs. Cependant, ce type d'immunisation présente l'inconvénient potentiel de diluer ou de dévier la réponse immune protectrice contre des antigènes non-protecteurs. Par conséquent, et dans le cas où l'antigène protecteur est connu, il est souvent plus simple et plus efficace de focaliser la réponse immune contre cet antigène défini (Titball, 2008). Un vaccin sous-unitaire est obtenu soit par purification directe de sous-unités bactériennes à partir de cultures de bactéries cibles, soit par la production d'antigènes recombinants. Ces derniers peuvent être produits par clonage du gène bactérien et exprimés dans un système bactérien ou eucaryote, puis purifiés, ou encore exprimés *in situ* par un vecteur vivant ou via l'intégration d'ADN nu (vaccin ADN) par les cellules de l'organisme hôte vacciné. Comme les vaccins inactivés, les vaccins sous-unitaires nécessitent généralement l'utilisation d'adjuvants ainsi que de multiples injections de rappels afin d'amplifier la réponse.

On peut citer, parmi les antigènes couramment ciblés, plusieurs adhésines de *S. aureus*. Celles-ci jouent un rôle essentiel dans la colonisation et l'infection de l'hôte. En effet, ces protéines de surface sont impliquées à la fois dans l'adhésion à différentes structures de l'hôte (cellules et matrice extracellulaire) et l'évasion des défenses immunitaires (Zecconi et Scali, 2013). En plus de la protéine A, qui lie fortement la partie constante des IgGs, elles incluent les *clumping factors* (CLfs) A et B, les protéines de liaison à la fibronectine (FnBPs) A et B, au fibrinogène (FgBP) et au collagène (CNA). Plusieurs études chez la souris ont démontré de bonnes réponses humorales spécifiques contre chacune de ces adhésines (Middleton, 2008). Cependant, l'immunisation contre celles-ci ne permet pas toujours de réduire la charge bactérienne des glandes mammaires et de protéger les souris contre l'infection expérimentale à *S. aureus* (Castagliuolo et al., 2006; Therrien et al., 2007).

L'obtention d'anticorps neutralisants contre les diverses toxines produites par *S. aureus* est une autre approche vaccinale intéressante. Chez l'homme, cibler les toxines par l'immunisation a pu générer des résultats prometteurs contre les pneumonies à *S. aureus*, entre autres (Spaulding et al., 2014). De manière générale, les vaccins à base de toxoïdes (toxines modifiées pour être inactives) qui ont été mis à l'essai dans le cadre de mammites à *S. aureus* ont visé principalement trois toxines : l'hémolysine-α (Hla) (Han et Park, 2000), l'entérotoxine-C (SeC) (Chang et al., 2008; Cui et al., 2010) et la TSST-1 (Cui et al., 2005; Hu et al., 2003). Malheureusement, malgré le développement de réponses humorales fortes et soutenues, aucune de ces études n'a encore mené à l'obtention d'une protection réellement significative chez la vache (Rainard et al., 2018). En effet, bien que les anticorps dirigés contre les toxines puissent être efficaces pour diminuer la sévérité clinique d'une infection, il est peu probable qu'ils soient suffisants pour protéger efficacement contre les nouvelles IIMs. L'étude de Cui *et al*. de 2010 a pu montrer une diminution significative des CCS des vaches vaccinées avec la toxine SeC mutée par rapport aux groupes n'ayant reçu que l'adjuvant, mais le peu d'animaux utilisés et le manque d'information sur la charge bactérienne subsistante au *challenge* ne permettent pas de tirer de conclusions claires sur son potentiel protecteur.

1.3.4.3 Cibles polysaccharidiques

Polysaccarides capsulaires

Bien que quantité de sérotypes polysaccaridiques capsulaires ont été décrits dans la littérature, la plupart des souches humaines de *S. aureus* expriment une capsule de type CP5 ou CP8. Cependant, environ 8% à 30% des isolats humains et jusqu'à 86% des isolats bovins (Grunert et al., 2013) n'expriment ni CP5 ni CP8. Les polysaccharides sont connus pour être faiblement immunogènes et indépendants des lymphocytes T (Kanswal et al., 2011) surtout dans le contexte de l'immunisation très ciblée des vaccins sous-unitaires. Afin d'augmenter leur immunogénicité, il est habituel d'utiliser des adjuvants et de les conjuguer à une protéine transporteuse afin d'induire une réponse croisée. Par exemple, on a pu démontrer que la conjugaison d'un polysaccharide capsulaire CP5 avec l'albumine sérique humaine permettait l'induction d'une réponse humorale spécifique et durable; cependant, les animaux vaccinés avec une souche complète inactivée exprimant le CP5 ont présenté un titre en anticorps spécifiques supérieur à ceux observés dans le groupe du vaccin conjugué (Tollersrud et al., 2001). On peut aussi mentionner les travaux portant sur le développement de StaphVAX® (Nabi Biopharmaceuticals) contre les infections humaines à *S. aureus*. Sa formulation consiste en l'association de deux polysaccharides capsulaires (CP5 et CP8) conjugués à une exotoxine A mutée. Elle a été démontrée comme étant sûre et immunogène, bien que l'immunisation de patients hémodialysés n'ait pas permis de mettre en évidence une réduction significative des bactériémies à *S. aureus* par rapport aux patients contrôles (Fattom et al., 2004). On a suggéré que la survie intracellulaire de *S. aureus* pourrait être impliquée dans l'absence de protection observée lors d'immunisation contre ces polysaccharides capsulaires (DeLeo et Otto, 2008).

Donc, pour obtenir de meilleures réponses contre les composantes polysaccharidiques de *S. aureus*, il semblerait que l'utilisation de vaccins plus complexes mettant à l'avant plan d'autres antigènes (tels que des cellules complètes) plutôt que seule des composantes de la capsule (qui

plus est, semble varier considérablement entre les souches associées à la mammite) serait plus efficace.

Biofilms

Les biofilms sont des agglomérats complexes de cellules bactériennes, de matrice organique et d'eau. Ces interfaces biologiques favorisent l'adhésion à plusieurs surfaces et offrent un environnement protecteur contre les défenses de l'hôte, les antibiotiques et une certaine tolérance à l'égard des substances biocides (Stewart, 2002, 2015). Autre que l'eau, la matrice organique représente la principale composante du biofilm et est composée principalement d'exopolysaccharides, de protéines (surfactantes, structurelles et enzymatiques), d'ADN extracellulaire et de lipides (Flemming et Wingender, 2010). Un grand nombre de bactéries, incluant différentes espèces et souches de staphylocoques, sont capables de produire du biofilm (Hall-Stoodley et al., 2004).

On considère de façon générale que la formation du biofilm suit trois phases distinctes, soit l'attachement, la maturation et la dispersion. La fixation des cellules de bactéries à une surface abiotique est permise principalement par des interactions hydrophobes ou électrostatiques, tandis que l'adhérence à une surface biotique est facilitée par les adhésines associées à la paroi cellulaire. La maturation comprend à la fois des processus d'adhésion qui fixent les bactéries entre elles et de désorganisation/dislocation qui forment des canaux dans la structure. Ces derniers sont essentiels pour le transport des éléments nutritifs dans les couches plus profondes du biofilm. En outre, la dispersion représente une troisième étape cruciale pour la propagation bactérienne lors du processus d'infection (Otto, 2008, 2013). Les molécules d'intérêt impliquées dans la formation de biofilm par *S. aureus* sont la *polysaccharide intercellular adhesin* (PIA), également appelé poly-N-acétyl-β-(1-6)-glucosamine (PNAG), différentes adhésines protéiques, l'acide téichoïque, et l'ADN extracellulaire (Otto, 2008). Parmi ces molécules, le PNAG représente l'un des facteurs de virulence les plus étudiés en raison de son rôle assez important dans la formation de biofilm. Diverses protéines, codées par le locus *ica* (*icaABCD*),

sont impliquées dans sa production et son transport. Néanmoins, le PNAG ne semble pas être obligatoire pour la formation de biofilm, puisqu'il a été démontré que dans les souches dépourvues des gènes *ica*, et par conséquent incapables de synthétiser celui-ci, plusieurs adhésines et protéines sécrétées peuvent remplacer ses fonctions (Otto, 2013). Citons notamment la protéine Bap (Valle et al., 2012), dont la présence chez les souches cliniques est associée à une plus grande résistance aux antibiotiques et persistance de *S. aureus* dans la mammite (Cucarella et al., 2004). Selon les études et les zones géographiques, on a rapporté que la fréquence des isolats de *S. aureus* d'IIM bovine producteurs de biofilm variait de 41 à 80% (Scali et al., 2015).

Bien que les rôles du biofilm dans les IIMs aient soulevé un grand intérêt au cours des dernières années, peu de données sur la vaccination concernant les modèles de la mammite de ruminants *in vivo* sont disponibles. Le vaccin commercialisé StartVac®, comme on l'a vu en 1.3.4.1, a pour concept l'utilisation de souches fortes productrices de SAAC inactivées, et a pu obtenir des résultats mitigés (Prenafeta et al., 2010; Schukken et al., 2014). Dans le même ordre d'idée, il a été récemment démontré que l'utilisation d'un vaccin complet constitué de souches productrices de biofilms conférait une réponse humorale contre le PNAG supérieure à l'injection du polysaccharide purifié, ainsi qu'une protection supérieure contre l'infection expérimentale de brebis par une souche productrice de biofilms (Pérez et al., 2009). Cela semble démontrer encore une fois que les bactéries complètes possèdent déjà un potentiel immunogène difficile à imiter avec des composantes isolées, même en présence d'un adjuvant. Une étude récente très intéressante chez la souris a par ailleurs pu comparer l'immunogénicité et le potentiel de protection de bactéries complètes inactivées issues d'un biofilm versus des cellules planctoniques en utilisant l'IIM murine comme modèle. Bien que le vaccin planctonique administré par voie sous-cutanée ait produit une réponse humorale nettement plus élevée, le vaccin contre le biofilm a pu obtenir de meilleurs résultats en tenant compte du niveau de protection par rapport à la réponse inflammatoire lorsqu'il fut administré par voie intramammaire (Gogoi-Tiwari et al., 2015).

1.3.4.4 Bactéries atténuées

Au cours des dernières décennies, quelques équipes ont mis au point des vaccins à base de bactéries vivantes atténuées contre *S. aureus* dans le contexte de la mammite bovine. Dès les années 80, Watson a décrit une souche atténuée de *S. aureus* utilisée pour vacciner des génisses (Watson, 1984). Ce type de vaccination présente l'avantage de mimer une infection naturelle et d'inclure l'ensemble des éléments clés nécessaires à l'induction d'une réponse immune adaptative, en agissant de façon intrinsèque comme adjuvant. Différentes techniques, physico-chimiques ou génétiques, permettent d'atténuer la virulence de pathogènes vivants tout en conservant le pouvoir immunogène. La prochaine section de ce document nous permettra d'entrer plus en profondeur dans ce sujet, et d'explorer les différents éléments à prendre en considération pour le développement d'un tel vaccin.

1.4 Les vaccins vivants atténués

Les vaccins vivants ont joué un rôle critique dans les tous débuts de la vaccinologie. En effet, la première expérience connue de vaccination fut l'inoculation en 1796 d'un jeune garçon par Edward Jenner avec des pustules de vache infectée par le virus de la vaccine (variole de la vache, *smallpox*), réputé pour causer des symptômes bénins chez l'homme, afin de le protéger contre celui de la variole, mortelle (De Gregorio et Rappuoli, 2014). Les nouvelles techniques immunologiques et biomoléculaires des dernières décennies ont permis un intérêt renouvelé et un immense essor de la recherche sur les vaccins vivants. On se penchera ici sur différents aspects liés au développement en général de vaccins vivants efficaces et sur leurs nombreuses applications, en mettant l'emphase sur les vaccins à base de pathogènes atténués et de possibles applications dans la recherche de protection contre *S. aureus*. Finalement, un survol des plus récents essais effectués dans le contexte de la mammite bovine et des perspectives d'utilisation d'une souche atténuée de *S. aureus* pour la vaccination contre les IIMs sera fait.

1.4.1 Caractéristiques des vaccins vivants

Les vaccins vivants atténués sont encore de nos jours parmi les technologies de vaccination les plus utilisées. Les vaccins atténués sont constitués de souches bactériennes ou virales, qui sont affaiblies par des mutations stables leur permettant de coloniser l'hôte cible de façon transitoire. Cette colonisation et multiplication transitoire déclenche une réponse immunitaire, sans provoquer les symptômes de l'infection associés à l'agent pathogène de type sauvage. Il existe un certain nombre d'avantages des vaccins vivants par rapport aux formulations tuées ou aux vaccins sous-unitaires: (i) ils imitent une infection naturelle, par conséquent peuvent provoquer des réponses immunitaires qui sont spécifiques, localisées, efficaces et de longue durée (Detmer and Glenting, 2006; Kollaritsch et al., 2000), (ii) ils peuvent prévenir l'infection par le pathogène, et non seulement les symptômes de la maladie (Frey, 2007), et (iii) en comparaison aux vaccins sous-unitaires hautement purifiés, ils sont relativement peu couteux à produire et à administrer. De plus, on peut les utiliser comme vecteurs vivants antigéniques *in situ*, pour exprimer des protéines hétérologues ou modifiées via des systèmes d'expression plasmidiques ou chromosomales. Ce type de vaccination présente en outre l'avantage non négligeable d'agir de façon intrinsèque comme un adjuvant naturel, de par sa stimulation des récepteurs de reconnaissance de motifs moléculaires (PRR) (Becker et al., 2008; Griffiths et Khader, 2014) permettant ainsi d'orienter et/ou d'augmenter la réponse au vaccin.

Bien qu'efficace, la technologie peut aussi poser des problèmes de sécurité liés au risque bien présent de réversion à un organisme virulent et la potentialité de provoquer la maladie chez des individus immunodéprimées (Zeman et al., 1993). Heureusement, au cours des 20 dernières années, les vaccins vivants ont regagné de l'intérêt en raison de notre compréhension immunologique accrue, de la disponibilité des séquences génomiques et des techniques moléculaires avancées, amenant ainsi la possibilité de vaccins vivants plus sûrs. Cela ouvre également la voie pour le développement de nouvelles applications aux vecteurs vivants bactériens qui sont liées à leur capacité de véhiculer des molécules aussi diverses que des

protéines conjuguées à des haptènes polysaccharides, des ADNs eucaryotes, et des antigènes anti-tumoraux (Galen et Curtiss, 2014).

1.4.2 Stimulation de l'immunité mucosale

Un autre avantage non-négligeable des vaccins vivants par rapport aux autres formulations réside dans leur voie d'administration et du type de réponse immune particulier que celle-ci permet d'éliciter. En effet, plusieurs vaccins vivants peuvent être administrés par voie orale, ou même nasale, offrant tout d'abord un meilleur profil de sécurité et une meilleure acceptation sociale que l'injection avec une seringue et une aiguille, et permettant de surcroit de stimuler la réponse immunitaire mucosale en plus de la réponse systémique.

L'immunité mucosale constitue une composante primordiale de la première ligne de défense d'un organisme, puisque la majorité des pathogènes entrent, causent des symptômes et sont reconnus via les tissus des muqueuses (Dietrich et al., 2003; Dwivedy et Aich, 2011). La reconnaissance des pathogènes est généralement initiée dans des sites inductifs tels que les plaques de Peyer dans le tissu lymphoïde gastro-intestinal et dans des sites équivalents dans le tissu des muqueuses nasales et les autres muqueuses des glandes périphériques. Cela est le cas comme nous l'avons vu pour *S. aureus* dans la glande mammaire (Bharathan and Mullarky, 2011).

Les composantes mucosales joueraient aussi un rôle central dans la régulation du SI distal des sites inductifs (McDermott et Huffnagle, 2014) et dans l'établissement d'une protection efficace et durable contre les pathogènes (Holmgren et Czerkinsky, 2005), en raison de son action à la fois localisée, spécialisée et polyvalente. Les vaccins d'administration mucosale peuvent activer d'un même front chaque bras effecteur du SI. Ils peuvent induire la production d'IgAs sécrétoires qui empêchent l'attachement et l'invasion et neutralisent les entérotoxines au site de colonisation, et des IgGs sériques qui contrôlent les pathogènes invasifs et leur dissémination systémique. Ils peuvent également promouvoir l'IMC contre les bactéries intracellulaires et les

virus ainsi que des réponses de cytotoxicité cellulaire anticorps-dépendante (Pasetti et al., 2011). Dans le contexte de la mammite, l'immunité mucosale prend une importance particulière car la glande mammaire fait partie des glandes exocrines prises en charge par celle-ci. L'infiltration de cellules immunitaires de la périphérie dans le lait pour combattre les infections est permise par la présence de chimiokines sécrétées par un niveau basal de cellules de «surveillance» dans le lait (Bharathan et Mullarky, 2011). L'IgG1 est l'isotype d'immunoglobuline principalement produit aux muqueuses chez les ruminants (contrairement à l'IgA chez l'homme ou le porc), et est transmis dans le lait pour protéger les petits de façon passive jusqu'au sevrage (Salmon, 1999).

1.4.3 Stratégies d'atténuation et équilibre immunogénicité/réactivité

Un point crucial et particulièrement ardu dans le développement d'un vaccin vivant atténué efficace et sécuritaire réside dans l'application des technologies de génie génétique en prenant un soin particulier à trouver le bon équilibre entre l'atténuation et la réponse immunitaire. L'atténuation doit évidemment être suffisante pour que le vaccin soit sécuritaire, mais une légère sur-atténuation, parfois seulement par l'ajout de l'expression hétérologue d'antigènes, peut facilement rendre le vaccin inopérant (Wang et al., 2013). Sans un examen soucieux des conséquences de l'atténuation sur le fitness métabolique de la souche et sa résistance au stress de l'hôte, on peut se retrouver avec des candidats présentant d'excellentes caractéristiques de sécurité, mais qui ont perdu soit la capacité de coloniser efficacement les sites d'induction immunologiques, soit celle d'exprimer l'antigène hétérologue, et par conséquent ne parviennent pas à induire une immunité protectrice (Galen et Curtiss, 2014; Gunn et al., 2010).

Traditionnellement, les vaccins vivants atténués ont été développés par le passage des agents pathogènes dans des conditions *in vitro* jusqu'à ce qu'ils perdent leur virulence chez l'homme. Cette approche empirique a été employée dans le cas de la souche vaccinale de *M. bovis* BCG. On considère maintenant que des stratégies d'atténuation dirigées, employant plusieurs inactivations affectant à la fois les facteurs de virulence et le métabolisme sont à préconiser

(Galen et Curtis, 2014). Les délétions complètes de gènes, comparativement aux mutagénèses de sites uniques, sont considérées plus sûres en raison de l'impossibilité de réversion, bien que l'acquisition de gènes de virulence fonctionnels complets par transfert horizontal via les bactéries de l'environnement est théoriquement possible (Frey, 2007).

1.4.4 Orientation de la réponse immune par les vaccins vivants

De nombreux pathogènes intracellulaires, tels que *Listeria monocytogenes*, ont pu non seulement être utilisés pour leur qualités de vaccin vivant atténués, mais également pour leur capacité d'échapper au phagolysosome lors de la phagocytose et ainsi de se retrouver dans le cytoplasme des cellules de l'hôte infecté (Bruhn et al., 2007). En effet, cette qualité permet la prise en charge des antigènes produits par la bactérie à la fois par le CMH de classe I et le CMH de classe II, puisque les antigènes sont produits directement dans le cytoplasme.

Comme le montre la figure 1.6, les voies « classiques » (A) de présentation sont sollicitées lorsque les cellules présentatrices d'antigène (CPA) sont directement infectées par un pathogène intracellulaire (figure 1.6A). Les antigènes des bactéries dégradées dans le phagolysosome sont acheminés via la voie du CMH-II, alors que ceux des bactéries échappées dans le cytoplasme sont pris en charge par le CMH-I. Un autre type d'admission des antigènes, la réception croisée (figure 1.6B), permet également de solliciter les deux voies, mais via une autre cellule infectée par le pathogène intracellulaire (dans la figure, un macrophage). Les antigènes se retrouvent ainsi excrétés dans des vacuoles ou sous forme libre pour être reçus par la cellule présentatrice d'antigène (CPA) et pris en charge par les deux types de CMH (Bruhn et al., 2007).

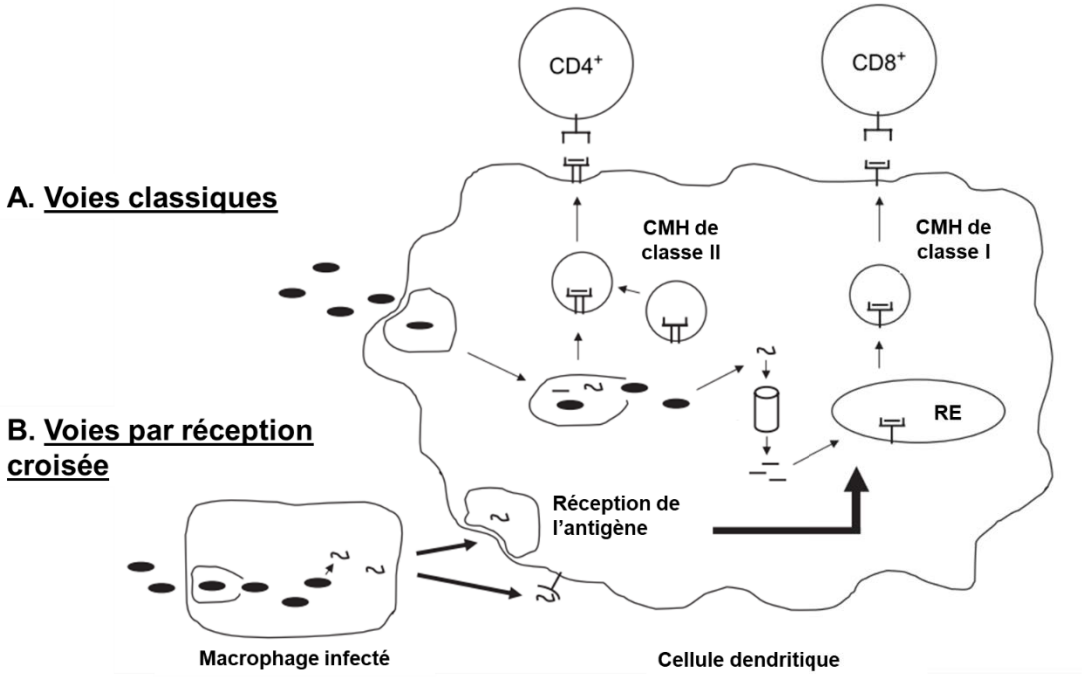

Figure 1.6. Voies de présentation antigénique. Le CMH I est utilisé pour la présentation aux cellules T CD8+ et le CMH II pour la présentation aux cellules CD4+. Les bactéries phagocytées sont dégradées dans les compartiments phagolysosomaux et les fragments de protéines sont dirigés dans des vésicules contenant le CMH de classe II pour le chargement et la présentation aux cellules T CD4+(A). Les bactéries capables de s'échapper dans le cytoplasme sécrètent des protéines qui sont traitées par la machinerie protéosomale cytosolique, et les peptides sont transportés dans le réticulum endoplasmique (RE) pour être chargés sur les molécules du CMH de classe I et présentés aux cellules T CD8+. (B) Les protéines bactériennes provenant de macrophages ou d'autres types de cellules infectées peuvent être acquises par les cellules dendritiques par le biais de divers mécanismes (phagocytose, endocytose médiée par les récepteurs, etc.) et transférées dans les voies de classe II et de classe I. Ces autres moyens d'acquérir et de présenter l'antigène sont appelés « présentation croisée » (adapté de Bruhn *et al.,* 2007).

En fonction des signaux captés en périphérie, les CPA, comme les cellules dendritiques, peuvent sécréter différentes cytokines qui orienteront les lymphocytes T vers un profil de différenciation spécifique (figure 1.7). Ainsi, les vecteurs antigéniques intracellulaires permettent de stimuler à la fois des fonctions effectrices de l'immunité humorale (la production d'anticorps par les lymphocytes B) et de l'immunité à médiation cellulaire (IMC), comme l'activation des lymphocytes T auxiliaires (Th) pour orchestrer la réponse adaptative (lymphocytes Th1, Th17 et Th2) ou pour tuer directement les cellules infectées (CTL; *cytotoxic T lymphocyte*) (figure 1.7).

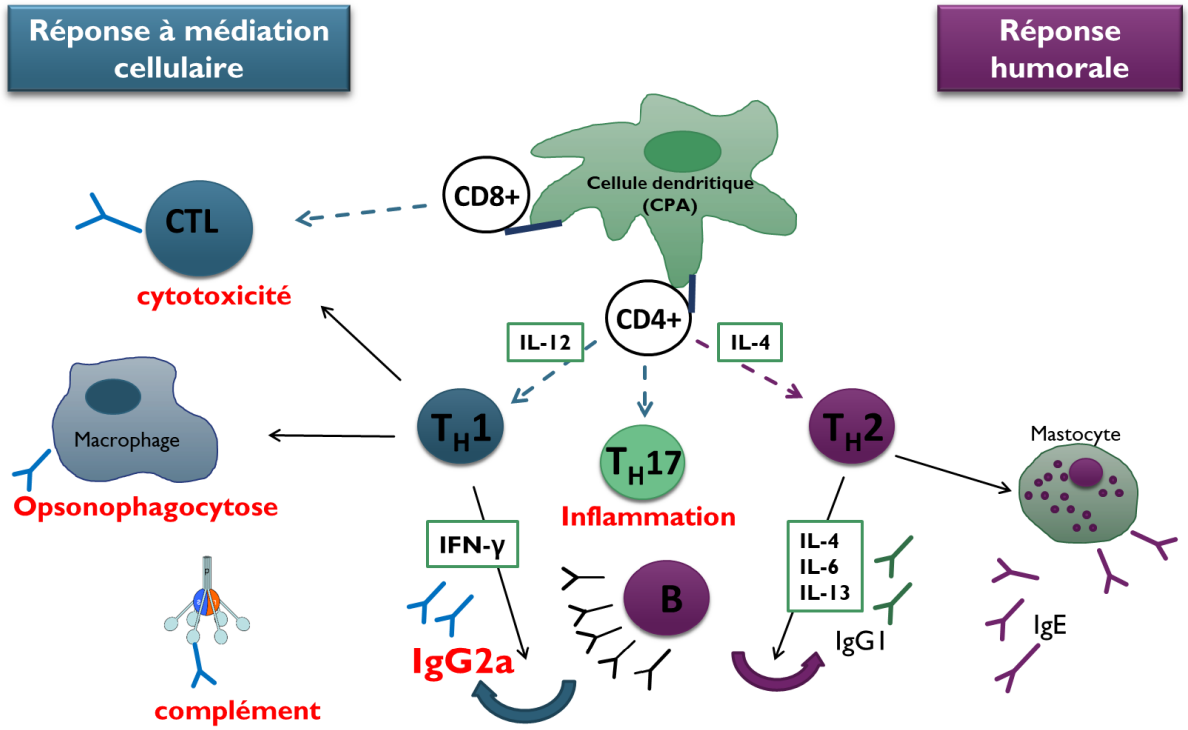

Figure 1.7. Orientation de la réponse immune. Les cellules dendritiques peuvent sécréter diverses cytokines qui guident les lymphocytes T vers un profil de différenciation particulier en fonction des signaux obtenus à leur périphérie**:** en réponse à la sécrétion d'IL-12 par les cellules

dendritiques, les cellules T CD4+ naïves se différencient en cellules Th1 productrices d'IFN-γ. On peut observer sur la figure que la voie Th1 favorisera la réponse cytotoxique (activation des CTL et de la phagocytose) alors que la voie Th2 stimulera fortement la production d'immunoglobulines E et G neutralisantes et par les lymphocytes B. Les isotypes et leur fonction effectrice générale sont illustrés pour la souris, et peuvent varier selon l'organisme. (LB: lymphocyte B; CTL: lymphocyte T cytotoxique; CPA : cellule présentatrice d'antigènes).

1.4.4.1 Importance de l'immunité à médiation cellulaire pour la protection contre *S. aureus*

De façon générale, il est actuellement suggéré qu'un vaccin permettant le développement d'une immunité plus équilibrée, avec une forte composante de l'IMC, soit nécessaire dans le cas de pathogènes responsables d'infections chroniques (Kovacs-Nolan et al., 2009). Dans le cas de l'homme, il est suggéré que puisque *S. aureus* fait partie de la flore normale de la peau et du nez, il est difficile de développer une immunité protectrice contre un pathogène qui a évolué depuis des milliers d'années pour contrecarrer le SI humain (Fowler et Proctor, 2014). Basé sur l'analyse d'essais cliniques de plusieurs vaccins, du niveau de protection obtenus avec ceux-ci, et de l'étude des effets des maladies génétiques de l'immunité sur le dénouement des infections à *S. aureus*, ces auteurs ont pu mettre en évidence l'importance de la réponse immune médiée par les lymphocytes, notamment de type Th-1 et Th-17, dans l'immunité contre *S. aureus*. La lignée de lymphocytes Th-17, plus récemment caractérisée, est maintenant considérée comme un acteur important dans la mobilisation de neutrophiles et la modulation de l'inflammation antigène-spécifique et innée (Weaver et al., 2006). Tel que discuté précédemment, *S. aureus* a la capacité de survivre dans les cellules de l'hôte : cela complique énormément le développement d'une immunité protectrice, et pourrait donc être une piste d'explication de l'inefficacité d'une réponse basée sur des anticorps. Ainsi, on se penche actuellement sur de nouveaux moyens de stimuler spécifiquement la voie Th-1 et Th-17 dans le développement de vaccins contre *S. aureus*.

Chez la vache laitière, on considère qu'une réponse plus équilibrée permettant l'induction de la voie Th1, caractérisée par l'activation des lymphocytes T cytotoxiques (CTL), l'adhésion des macrophages et PMNs, la diapédèse et l'opsonisation, pourrait être plus efficace pour protéger la glande mammaire contre un pathogène chronique tel que *S. aureus* (Gaudreau et al., 2007; Osogne et al., 2002). La voie Th-17 a également été mise de l'avant : on a pu montrer qu'elle pouvait être induite directement dans la glande mammaire, en caractérisant des réponses inflammatoires antigènes spécifiques Th-1 et Th-17 suite à l'immunisation intramammaire de vaches avec une protéine sensibilisante (Rainard et al., 2013). L'utilisation d'un vaccin vivant à base d'une souche atténuée de *S. aureus* pourrait ainsi être une approche intéressante pour obtenir ce type de réponse plus balancée vers l'IMC.

1.4.5 Exemples de souches atténués de *S. aureus* pour la vaccination homologue

Peu d'études on fait état de tentatives d'utilisation de *S. aureus* comme vaccin vivant chez l'humain. Tel que brièvement discuté précédemment dans ce document, quelques équipes ont mis au point des vaccins atténués contre *S. aureus* pour la mammite bovine depuis la première caractérisation d'une souche atténuée (Watson, 1984). Un groupe a pu mettre au point un vaccin atténué par mutagénèse chimique. La souche atténuée RC122, issue de la souche pathogène RC108, a été mise à l'essai dans un *challenge* de 8 vaches par Pellegrino *et al*. en 2008. Après l'immunisation de vaches vaccinées à l'aide de cette souche RC122, une augmentation significative de la réponse immune humorale spécifique a été observée par rapport au groupe d'animaux non immunisés, à la fois dans le lait et le sang. Cependant, après l'infection expérimentale à l'aide de la souche virulente RC108, la comparaison des comptes bactériens et des CCS moyens dans le lait n'a pu révéler de différence significative entre les vaches vaccinées et contrôles (Pellegrino et al., 2008). L'étude fut reprise en 2010 avec de nouvelles conditions expérimentales, notamment en réduisant le nombre d'UFCs de *S. aureus* du *challenge* de 10^3 à $2.5x10^2$ (Pellegrino et al., 2010). Une légère réduction de la charge bactérienne a pu être obtenue dans le groupe vacciné, mais encore une fois, avec peu d'animaux (5 vaccinés, 4 contrôles). Malheureusement, l'administration sous-cutanée du vaccin pourrait ne pas être appropriée au

développement d'une immunité protectrice, puisque le principal paramètre amélioré par le vaccin fut une plus grande réponse IgG du groupe vacciné. Pourtant, une certaine diminution des symptômes cliniques a effectivement pu être observée (Pellegrino et al., 2010). Par ailleurs, le même groupe a pu obtenir une autre souche de *S. aureus* atténuée par mutagenèse du gène *aroA*, la rendant auxotrophe pour les acides aminés aromatiques. L'immunisation intramammaire de souris par ce mutant a permis d'induire une réponse mixte T-auxiliaire Th1 et Th2, et de réduire significativement les comptes bactériens de souris infectées expérimentalement par *S. aureus* (Buzzola et al., 2006).

Des bactéries commensales ont aussi déjà été utilisées pour délivrer des antigènes hétérologues de *S. aureus*. Dans une étude d'Asensi et al. (2013), une séquence de gènes codant pour une toxine SEB de *S. aureus* recombinante (*rseB*), dépourvue d'activité superantigénique, a été clonée et exprimée dans le cytoplasme ou sous une forme sécrétée par *L. lactis.* L'immunisation par voie orale avec les souches recombinantes de *L. lactis* a pu induire dans les deux cas une réponse immunitaire protectrice dans un modèle murin d'infection à *S. aureus* lors d'un *challenge* intrapéritonéal avec une souche productrice de SEB.

1.5 Travaux antérieurs : gènes de virulence associés à l'IIM d'intérêt pour la vaccination

Certaines études qui ont précédemment été effectuées au laboratoire doivent être mises de l'avant pour la compréhension du contexte de ce projet de doctorat. Comme mentionné précédemment dans ce document, l'équipe du Pr. Malouin a pu mettre en évidence une trentaine de gènes de *S. aureus* exprimés de façon importante et soutenue par différentes souches à l'aide d'analyses transcriptomiques au cours d'IIMs expérimentales chez plusieurs vaches (Allard et al., 2013). L'équipe a entre autres démontré pour la première fois l'importance du gène surexprimé SACOL0720 (*vraG*) dans la virulence de *S. aureus in vivo*, via l'atténuation importante du mutant de délétion Δ*vraG* lors d'une autre IIM expérimentale (Allard et al., 2013). Comme on l'a vu dans la section 1.2.3, *vraG* avait déjà été décrit pour son rôle dans la résistance à certains CAMPs de l'hôte (Falord et al., 2012) et la résistance intermédiaire à la vancomycine

(Meehl et al., 2007). L'importance *in vivo* de ce gène, rapportée pour la première fois par notre équipe, démontrait ainsi son potentiel pour la vaccination ou l'atténuation.

Le gène *vraG* (SACOL0720), avec 5 autres gènes surexprimés *in vivo*, ont été testés sous forme de vaccin protéique sous-unitaire pour leur habilité à protéger des vaches laitières suite à une infection expérimentale à *S. aureus*. Le vaccin sous-unitaire a pu induire une forte réponse contre les six antigènes chez les vaches vaccinées, à différentes intensités selon l'antigène. Les vaches vaccinées ont également montré un compte de cellules somatiques (CCS) significativement inférieur comparativement aux vaches du groupe témoin suite à l'infection par *S. aureus*. Ce nouveau vaccin a également récemment été mis à l'essai par notre équipe en le comparant directement au vaccin commercial STARTVAC®, et s'est démontré supérieur de façon significative suite aux infections expérimentales, tant au niveau de la production de lait (supérieure pour les vaches vaccinées UdeS) que des comptes bactériens dans le lait (Ster et al., en préparation).

Parallèlement aux essais du vaccin sous-unitaire chez la vache, le développement d'un vaccin vivant atténué basé sur une souche SCV génétiquement stable de *S. aureus* a été amorcé lors de mes études de maîtrise dans le but à la fois 1) d'offrir une formulation vaccinale plus susceptible de stimuler une réponse ciblant les phénotypes persistants de *S. aureus* et 2) d'augmenter et de moduler vers la médiation cellulaire la réponse contre les différents antigènes du vaccin sous-unitaire. L'hypothèse générale de ce projet suggérait que le phénotype SCV de *S. aureus* peut être utilisé comme squelette de base d'une souche atténuée suite à une stabilisation génétique du phénotype par la délétion complète du gène *hemB*. Puisque le phénotype SCV est caractérisé par une réduction de l'expression des facteurs de virulence invasifs (α-hémolysine, coagulase, etc.) par rapport au phénotype régulier, ce squelette de base (i.e. SCV) permettait à la fois une croissance diminuée et une certaine atténuation de la virulence.

J'ai ainsi précédemment effectué la construction d'une souche atténuée de *S. aureus* par la délétion irréversible du gène *hemB* (Δ*hemB*) pour l'obtention d'un SCV stable. De plus, une

atténuation supplémentaire a pu être obtenue par l'inactivation du gène *vraG*, qui comme on l'a vu est important pour la virulence pendant l'IIM chez le bovin (Allard et al., 2013). Pour établir la preuve de concept qu'une telle souche peut effectivement être sécuritaire pour la vaccination, la caractérisation et l'évaluation de son atténuation dans des modèles de culture cellulaire et *in vivo* chez la souris pour confirmer son innocuité est l'approche qui a été sélectionnée. Les mutants isogéniques simples et double (souche atténuée) pour ces gènes ont été comparés à la souche parentale *wild type* (WT), ATCC 29213, dans des modèles d'infection *in vitro* et *in vivo*. Dans un modèle d'invasion de cellules épithéliales mammaires bovines (MAC-T), le double mutant Δ*vraG*Δ*hemB* s'est montré nettement atténué (figure 1.8A, B, C), avec une destruction cellulaire et une internalisation significativement plus faible par rapport à celles observées avec les mutants simples Δ720 (Δ*vraG*) et Δ*hemB*, respectivement. L'évaluation de la sécurité de la souche par l'inoculation intramammaire du double mutant dans un modèle de mammite murin a également pu révéler une atténuation avec une charge bactérienne fortement réduite (différence de 5 log10 UFC/g de glande par rapport au type sauvage), conduisant ultimement à des glandes exemptes de comptes bactériens détectables après 12 jours. À titre comparatif, la souche WT a provoqué des infections invasives aigues, en maintenant des comptes bactériens élevés (>9.5 log UFC/g de glande) et en entraînant la mort des souris dès 48 h post-infection (figure 1.8D).

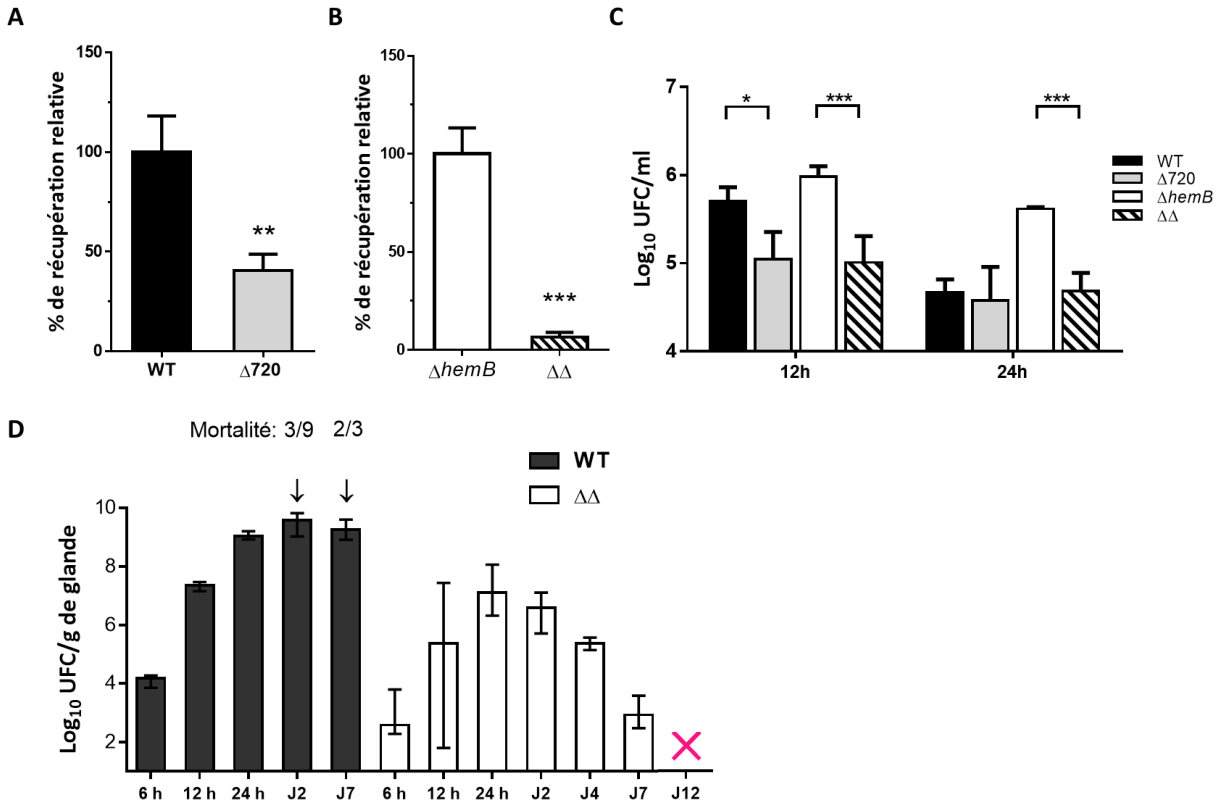

Figure 1.8. Atténuation de la souche Δ*vraG*Δ*hemB* dans les modèles d'infection de cellules MAC-T et pendant l'IIM murine. Influence de la mutation Δ*vraG* (Δ720) sur l'invasion (A et B) et la persistance (C) des souches isogéniques de *S. aureus* de phénotypes normales et SCV dans les cellules MAC-T. Les cellules ont été infectées avec les quatre souches pendant 3 heures à une MOI de 50, puis ont été incubées en présence de lysostaphine 30 min supplémentaire (t0), 12h, ou 24h (C), puis lysées. Récupération relative de l'inoculum initial dans les cellules à T0 pour les souches normales (A) et SCVs (B). Les résultats sont normalisés en fonction de ceux obtenus pour ATCC 29213 (WT) ou Δ*hemB*, respectivement (100%), et sont exprimées en moyennes avec la SD. (** : $P \leq 0.01$; *** : $P \leq 0.001$; test t non apparié). (C) Moyennes et SD des comptes intracellulaires d'UFC de bactéries de type sauvage et mutants à 12h (gauche) et 24h (droite). (Two-Way ANOVA et comparaisons multiples avec le test de Tukey : * : $P \leq 0.05$; ***: $P \leq 0.001$). (D) IIM murine avec la souche parentale (WT) et Δ720Δ*hemB* (ΔΔ). Les souris ont été infectées selon le modèle d'IIM murine précédemment décrit (Brouillette et al., 2004) et les glandes récoltées à l'heure indiquée (h) ou le jour (J) après l'infection. Chaque colonne représente la valeur médiane des comptes d'UFC pour un groupe de glandes, et la portée

des valeurs est indiquée par des barres. La mortalité des souris au moment spécifique pointé est indiquée par des flèches. Le X rouge indique l'absence de bactéries détectables (Côté-Gravel et al., 2016).

Il est aussi important de noter que bien que l'infiltration des neutrophiles (évaluée par une activité myéloperoxydase (MPO) aussi élevée chez les souris infectées) dans les glandes a démontré être similaire à 24 h post-infection pour les souches mutante et WT, l'inflammation visuelle et la destruction tissulaire induite par cette dernière était nettement plus prononcée (Côté-Gravel et al., 2016). Cet effet inusité a ainsi pu démontrer que malgré le caractère explicitement moins invasif et virulent de la souche Δ*vraG*Δ*hemB*, celle-ci peut malgré tout coloniser la glande de façon transitoire et éliciter la stimulation du SI inné de la souris, en faisant un bon candidat pour l'élaboration d'un vaccin vivant.

Ainsi, cette étude a pu faire la démonstration qu'une souche atténuée à capacité intracellulaire pouvait être obtenue à partir de SCVs génétiquement stables. L'élimination complète de la souche via le recrutement efficace de cellules immunitaires de la réponse innée ont pu témoigner à la fois de l'innocuité de la souche chez la souris, et de premiers indices quant à sa possible immunogénicité. L'équilibre délicat entre l'atténuation et immunogénicité est en effet l'un des défis les plus ardus du développement de vaccins vivants atténués efficaces (Galen et Curtiss, 2014).

1.6 Hypothèse et objectifs de recherche

1.6.1 Hypothèse générale

L'hypothèse générale de cette thèse suggère que la présence de SCVs et/ou de phénotypes persistent dans l'IIM est une composante non négligeable de la pathogénèse de *S. aureus*, et peut expliquer en partie l'échec de protection actuellement obtenu lors de la vaccination et l'antibiothérapie. Ainsi, une protection efficace nécessiterait l'utilisation d'un vaccin imitant le

plus fidèlement possible une infection naturelle, et ainsi plus adaptée à développer spécifiquement une immunité protectrice de la glande mammaire. Nous proposons donc qu'un vaccin vivant atténué de *S. aureus* ayant un phénotype SCV peut être utilisé pour éliciter une réponse immune plus balancée vers la médiation cellulaire augmentant potentiellement ainsi l'efficacité de protection contre les IIMs, et que la forte atténuation d'une telle souche vaccinale (Δ*vraG*Δ*hemB*) s'explique en partie par le rôle important joué par le système *graXRS-vraFG* dans la persistance du SCV chez l'hôte.

1.6.2 Objectifs généraux

L'objectif général premier de cette étude consiste en la preuve de concept et la caractérisation de l'immunogénicité d'un vaccin vivant atténué au phénotype SCV chez la souris. En second lieu, l'étude doit permettre de déterminer les bases moléculaires de l'atténuation de la souche vaccinale SCV atténuée (Δ*vraG*Δ*hemB*) et de mieux comprendre le rôle et l'importance spécifique au phénotype SCV du système *graXRS-vraFG* dans la résistance aux peptides antimicrobiens et au stress oxydatif chez l'hôte.

1.6.3 Objectifs spécifiques

Cette section définie plus en détail les objectifs spécifiques de la thèse ainsi que les chapitres et sections de ce document où ceux-ci sont réalisés.

Objectif 1. Démontrer et caractériser la stimulation de la réponse immune chez la souris par le vaccin SCV atténué (Δ*vraG*Δ*hemB*) (preuve de concept) : **Chapitre 4** (section 4.2).

a) Caractériser la réponse humorale chez la souris via la vaccination par des doses croissantes de bactéries du vaccin SCV atténué;

b) Démontrer la reconnaissance de composants bactériens d'isolats cliniques de mammite par le sérum immun de souris vaccinées par le vaccin SCV atténué;
c) Comparer la réponse humorale de souris immunisées par le vaccin SCV atténué *vs* celle engendrée par des protéines de *S. aureus* purifiées (vaccin sous-unitaire).

Objectif 2. Démontrer l'avantage d'utiliser un vaccin SCV atténué en combinaison avec des protéines de *S. aureus* purifiées (vaccin sous-unitaire) pour améliorer la réponse immune contre ceux-ci (qualité « d'adjuvant ») : **Chapitre 4** (section 4.4).

a) Comparer les titres en anticorps spécifiques aux antigènes protéiques utilisés pour la vaccination lorsque formulés seuls ou en combinaison avec le vaccin SCV atténué;
b) Comparer la balance IgG2a/IgG1 (réponse Th-1/Th-2) spécifique aux antigènes protéiques utilisés pour la vaccination lorsque formulés seuls ou en combinaison avec le vaccin SCV atténué.

Objectif 3. Démontrer l'avantage du vaccin SCV atténué par rapport à une formulation de bactéries inactivées et comparer a) la réponse humorale et b) la réponse à médiation cellulaire induite chez la souris par le vaccin SCV atténué (**Chapitre 2**) *vs* :

i. Forme inactivée du vaccin SCV;
ii. En combinaison avec une souche *E. coli* J5 inactivée;
iii. Forme inactivée du vaccin SCV en combinaison avec une souche *E. coli* J5 inactivée

Objectif 4. Caractériser et définir les différences phénotypiques des quatre souches isogéniques (WT ATCC 29213 et ses mutants Δ*vraG,* Δ*hemB* et Δ*vraG*Δ*hemB*) pour comprendre la base de l'atténuation de la souche vaccinale et élucider l'importance du gène *vraG* et du phénotype SCV dans la résistance aux CAMPs (**Chapitre 3**) :

a) Comparer le profil de susceptibilité à différents antibiotiques et peptides antimicrobiens des souches isogéniques;
b) Comparer les propriétés de surface bactériennes, soit la charge nette de surface, l'hydrophobicité et le potentiel membranaire des quatre souches isogéniques.

Objectif 5. Comparer le profil transcriptionnel des quatre souches isogéniques (WT ATCC 29213 et ses mutants Δ*vraG,* Δ*hemB* et Δ*vraG*Δ*hemB*) en réponse à l'exposition au polypeptide antimicrobien colistine (**Chapitre 3**) :

a) Caractériser le profil d'expression génique par une étude transcriptomique (RNA-seq) de cultures des quatre souches isogéniques en absence ou présence du polypeptide cationique colistine.

CHAPITRE 2

VACCINATION WITH A LIVE-ATTENUATED SMALL-COLONY VARIANT IMPROVES THE HUMORAL AND CELL-MEDIATED RESPONSES AGAINST STAPHYLOCOCCUS AUREUS

2.1 Introduction de l'article et contribution des auteurs

Staphylococcus aureus est un pathogène responsable d'infections persistantes et chroniques chez les humains et les animaux, et les *small-colony variants* (SCV), qui produisent des niveaux plus élevés de biofilm et qui sont capables de persistance intracellulaire, contribuent à cette chronicité et à la récurrence des infections. La prévention des infections à *S. aureus* par la vaccination, notamment les infections intramammaires, n'a pour l'instant pas encore rencontré de succès considérable. Certaines des formulations de vaccins actuellement commercialisées contre la mammite bovine à *S. aureus* consistent en des bactéries inactivées, parfois associées à la souche *E. coli* J5, mais la stimulation de l'immunité à médiation cellulaire par ces formulations pourrait ne pas être optimale. Lors de mes études de maîtrise, j'avais conçu une souche SCV génétiquement stable (Δ*vraG*Δ*hemB*), qui avait démontré une forte atténuation dans un modèle d'infection intramammaire murin. Cet article s'insère dans la suite de ces travaux, et caractérise les réponses immunitaires suscitées chez la souris par diverses compositions vaccinales expérimentales dont ce SCV vivant atténué (Δ*vraG*Δ*hemB*) et sa forme inactivée, associés ou non à la souche *E. coli* J5 inactivée, afin de démontrer les avantages du vaccin vivant.

Pour cet article, j'ai moi-même effectué la grande majorité de la méthodologie, des expériences et des analyses, avec l'aide d'Eric Brouillette en particulier pour les étapes d'immunisation chez la souris. J'ai rédigé le manuscrit qui a par la suite été révisé par mon superviseur, Pr. François Malouin. Les trois auteurs ont participé à la conceptualisation des expériences.

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Vaccination with a live-attenuated small-colony variant improves the humoral and cell-mediated responses against *Staphylococcus aureus*

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2.2 Abstract

Staphylococcus aureus is known to produce persistent and chronic infections in both humans and animals. It is recognized that small-colony variants (SCVs), which produce higher levels of biofilm and that are capable of intracellular persistence, contribute to the chronicity or recurrence of infections and that this phenotype is inherent to the pathogenesis process. Prevention of *S. aureus* infections through vaccination has not yet met with considerable success. Some of the current vaccine formulations for *S. aureus* bovine mastitis consist of inactivated *S. aureus* bacteria, sometimes combined to *E. coli* J5. As such, the stimulation of cell-mediated immunity by these vaccines might not be optimal. With this in mind, we recently engineered a genetically stable double mutant SCV (*ΔvraGΔhemB*), which was highly attenuated in a mastitis model of infection. The present work describes the immune responses elicited in mice by various experimental vaccine compositions including the live-attenuated SCV double mutant and its inactivated form, combined or not with inactivated *E. coli* J5. The live-attenuated SCV was found to provoke a strong and balanced humoral response in immunized mice, as well as strong proliferation of *ex-vivo* stimulated splenocytes isolated from these animals. These splenocytes were also found to release high concentration of IL-17 and IFN-γ when compared to every other vaccination formulation. Inversely, the inactivated whole-cell vaccine, alone or in combination with the *E. coli* J5 bacterin, elicited lower antibody titers and failed to induce Th1 or Th17 cell-mediated responses in the splenocyte proliferation assay. Our results suggest that live-attenuated SCVs can trigger host immunity differently than inactivated bacteria and could represent a suitable vector for inducing strong humoral and cell-mediated immune responses, which are crucial for protection. This could represent an important improvement over existing vaccine formulations for preventing *S. aureus* bovine mastitis and other infections caused by this pathogen.

2.3 Introduction

Staphylococcus aureus is an opportunistic pathogen that has the ability to affect several tissues and organs in human and animal hosts, and to induce both acute and chronic types of infections. This pathogen possesses an abundance of virulence factors, with many of them contributing to its ability to persist in host cells and tissues, resist or counter drug therapies and evade host immune responses [1]. The development of new alternatives to fight this pathogen is becoming increasingly urgent. Vaccine development against *S. aureus* for either humans or animals has been unsuccessful to date. Challenges include the diversity of strains that can cause infections, the ability of *S. aureus* to counteract host immune defenses [2] and insufficient understanding of the type of immune defense required for efficient protection against such a polyvalent pathogen with both extracellular and intracellular lifestyles [3].

Staphylococcus aureus is the most commonly found pathogen in clinical bovine mastitis [4], but it is also the cause of subclinical, persistent and difficult-to-treat intramammary infections (IMIs) [5,6]. Bovine mastitis affects animal health, milk production and quality, and challenges the economic efficiency of dairy producers [7]. Spreading of undetected subclinical IMIs during milking maintains a reservoir in the herd and is a difficult problem that may be better tackled through preventive interventions. Vaccines could represent the ideal prevention tools to reduce the incidence of new cases of IMIs and improve milk production and quality.

Vaccine development for *S. aureus* mastitis is challenging [8]. Commercially available vaccines for the prevention of *S. aureus* mastitis consist of inactivated bacteria or bacterin-based products, including a lysed whole cell vaccine of capsular *S. aureus* serotypes (Lysigin, Boehringer Ingelheim Vetmedica, Inc.) [9] and a multivalent inactivated vaccine (StartVac® or TopVac®, Hipra, Spain) composed of *E. coli* J5 and a *S. aureus* strain that expresses slime-associated antigens part of the biofilm extracellular matrix [10]. Although the use of whole bacterins provides a selection of antigens that are suitable for raising an immune response, the success of such an approach is highly dependent on the diversity and type of *S. aureus* strains

present in herds. Additionally, it is still unclear if such multivalent inactivated vaccines have the ability to raise the adequate type of immune response to protect against *S. aureus* infections, as they have been shown to generate mostly humoral responses against this pathogen [11]. Antibody-based immunity may be important but is likely insufficient for protection against *S. aureus* chronic infections without the contribution of a cell-mediated response [12,13].

In human and veterinary medicine, *S. aureus* small-colony variants (SCVs) contribute to therapeutic failures and are frequently isolated from chronic infections [14,15]. SCVs are adapted for long-term persistence and are capable of high biofilm production [16,17] and invasion of host cells [18,19], shielding the bacteria from drugs and the host immune system. Several SCV isolates from dairy cattle with a history of chronic mastitis have been previously reported [20,21] but are usually overlooked in routine milk culture procedures because of their slow growth and atypical colony appearance. Recurrent antibiotic treatments and internalization of *S. aureus* in mammary gland epithelial cells may indeed represent favorable conditions to the generation of SCVs [20], and potentially explain some of the relatively low cure rates observed for *S. aureus* IMIs [22]. Hence, SCVs can add important contributions to the persistence in infections; however, their natural slow-growing phenotype and low expression of dissemination virulence factors could also be exploited in vaccines development, following further attenuation.

Genetically stable *S. aureus* SCVs can be engineered through the deletion of gene *hemB* [23] to prevent reversion to the virulent prototypic phenotype that expresses numerous exotoxins. In a previous work [24], we have constructed a double mutant by the complete deletion of *hemB* in addition to the inactivation of gene *vraG* (SACOL0720), which was shown to be important for full virulence during bovine IMIs [25]. The Δ*vraG*Δ*hemB* SCV strain was shown to be greatly attenuated in a bovine epithelial mammary cells invasion/persistence assay and in the murine intramammary infection (IMI) model [24]. Additionally, high doses of subcutaneous injections could be achieved in mice without provoking any sign of local inflammation or adverse effect. Such a strain could therefore be used as a live-attenuated vaccine. Immunization of mice using increasing concentrations of Δ*vra*GΔ*hemB* yielded a substantial rise of specific antibody titers

against a variety *S. aureus* strains isolated from bovine mastitis, including strains from the major *spa* types found in Canada and elsewhere in the world [24]. Live-attenuated vaccine that mimic natural infections are known to stimulate the immune system in a powerful way, producing high affinity serum and mucosal antibodies as well as different effectors of cell-mediated immunity due to the recognition of microbial viability by the innate immune system [26].

In the present work, we describe the characterization of the humoral and cellular responses that develop following vaccination with the live-attenuated Δ*vra*GΔ*hemB* SCV vaccine. These responses were compared to that achieved with its inactivated version, alone or in combination with inactivated *E. coli* J5 bacteria. *E. coli* J5 is a well-known O polysaccharide mutant, which exposes its lipopolysaccharide (LPS) core region and that has been used for producing cross-reacting antibodies against *Enterobacteriaceae* and Gram-negative bacteria [27]. This characterization could help to attain a better understanding of the factors behind the suboptimal protection currently achieved with vaccines that use inactivated *S. aureus* or that combine *S. aureus* antigens to Gram-negative bacterins. Results revealed the potential advantages of developing alternate strategies such as immunization with live-attenuated *S. aureus* strains, particularly in order to improve cell-mediated immunity and protection against *S. aureus*.

2.4 Materials and Methods

2.4.1 Ethics statement

The animal experiments were conducted following the guidelines of the Canadian Council on Animal Care and the institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke. The institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke approved this study.

2.4.2 Bacterial strains and *S. aureus* live-attenuated vaccine

Unless otherwise stated, *S. aureus* and *Escherichia coli* J5 strains were grown in tryptic soy broth (TSB) and agar (TSA) (BD, Mississauga, ON, Canada). The *E. coli* J5 strain was obtained from the American Type Culture Collection (ATCC 43745). The development of the *S. aureus* double mutant strain Δ*vraG*Δ*hemB* was described elsewhere [24]. For the preparation of bacterial vaccine samples, *S. aureus* Δ*vraG*Δ*hemB* colonies previously grown on brain heart infusion agar (BHIA) (BD) were washed twice in ice cold PBS (Wisent, St-Bruno, QC, Canada) and suspended in PBS containing 15% glycerol, then were aliquoted and kept at -80°C until subsequent use. The concentration of *S. aureus* Δ*vraG*Δ*hemB* was assessed by serial dilutions in PBS and plating on TSA, and suspensions were freshly adjusted to 5×10^7 CFU/ml of PBS on the immunization day.

2.4.3 Inactivation of bacteria

Bacterial suspensions of *S. aureus* Δ*vraG*Δ*hemB* and *E. coli* J5 were also heat-killed to obtain an inactivated version of the vaccines for immunization and for stimulation of mice splenocytes in subsequent assays. Different heat inactivation treatments were evaluated to select the lowest temperature and time exposure to attain total killing of bacteria. *S. aureus* Δ*vraG*Δ*hemB* and *E. coli* J5 previously grown and prepared in suspensions of 5×10^7 CFU/ml in PBS were treated for 10, 20 or 30 min at 65 °C and 5 min at 80 °C. Undiluted bacterial suspensions were then plated (200 µl) in triplicate on BHIA and incubated for 48 h at 37°C to confirm inactivation (no growth). For *E. coli* J5*,* 10 min at 65°C was found to be sufficient for complete inactivation of bacteria whereas 20 min at the same temperature was necessary for killing of *S. aureus* Δ*vraG*Δ*hemB*. Inactivated bacteria were stored at – 80°C until subsequent use.

2.4.4 Preparation of *S. aureus* cell extract

Preparation of a *S. aureus* Δ*vraG*Δ*hemB* whole cell extract was done as previously described with some modifications [28]. Briefly, overnight bacterial cultures were diluted 1/1000 in fresh BHI broth, and then incubated at 35°C (225 rpm) until an A_{600nm} of ~ 0.8 was reached. Bacterial cells were centrifuged, and pellets were washed twice in ice-cold PBS and resuspended in a ratio of 5 ml of PBS per ml of pellet. Bacterial suspensions were then treated with 100 µg of lysostaphin (Sigma-Aldrich, Oakville, ON, Canada) per ml of pellet for 1 h at 37°C, and then 3 µg of protease inhibitor cocktail (Sigma-Aldrich), 8 µg of RNAse A (Sigma-Aldrich) and 8 µg of DNAse (Qiagen, Toronto, ON, Canada) per ml of pellet were added to the suspension. After 30 min at room temperature, cells were mechanically disrupted by 3 to 4 passages in a SLM Aminco French Pressure cell disrupter, and then centrifuged at 12,000 × *g* at 4°C for 10 min to remove unbroken cells. The supernatant was collected and used as the whole cell extract. Total protein concentration was determined by the bicinchoninic acid method (BCA) Protein Assay Kit (Thermo Fisher Scientific, Ottawa, Canada).

2.4.5 Immunization of mice

CD-1 female mice weighing 16-18 g were obtained on demand from Charles River Laboratories Inc. (Saint-Constant, QC, Canada). After arrival at our animal facilities, 5 mice per filtered cage were randomly assigned, and had *ad libitum* access to food and water. Prior to each experimental injection or blood samplings, animals were anesthetized by intramuscular injection of a mixture of ketamine and xylazine at 87 and 13 mg per kg of body weight. Throughout the experimentation, animal health was daily monitored by a certified animal care technician. Mice were immunized by two subcutaneous injections (100 µl) two weeks apart, following the timeline illustrated in figure 2.1. CD-1 mice were divided into 6 groups (n = 5 mice per group): group 1 (SCV Inac) received the heat-inactivated *S. aureus* Δ*vraG*Δ*hemB* (5×10^7 CFU that were heat-killed); group 2 (SCV Live), received the live-attenuated *S. aureus* Δ*vraG*Δ*hemB* (5×10^7 CFU); group 3 (SCV Live 3), received the same regimen as group 2, but with an additional

boost immunization (3 injections in total) 2 weeks after the 2nd immunization as illustrated in figure 2.1; group 4 (SCV Inac + J5), received a combination of the heat-inactivated *S. aureus* Δ*vraG*Δ*hemB* and the heat-inactivated *E. coli* J5 (5×10^7 CFU of each heat-killed bacterial suspensions); group 5 (SCV Live + J5), received a combination of the live-attenuated *S. aureus* Δ*vraG*Δ*hemB* (5×10^7 CFU) and the heat-inactivated *E. coli* J5 (5×10^7 CFU); and group 6 (PBS), received 100 µl PBS. Blood samples were collected prior to the first injection and 10 days after the final boost. The blood samples were allowed to clot at room temperature for an hour and were then centrifuged at 2,000 × *g* for 10 min at 4°C. The sera were harvested and kept at -20°C until subsequent analysis. Ten days after the final boost, mice were euthanized by cervical dislocation after deep anesthesia with the ketamine and xylazine mixture (see above), and spleens were aseptically harvested to isolate fresh splenocytes intended for antigen-specific cell proliferation and cytokine production assays.

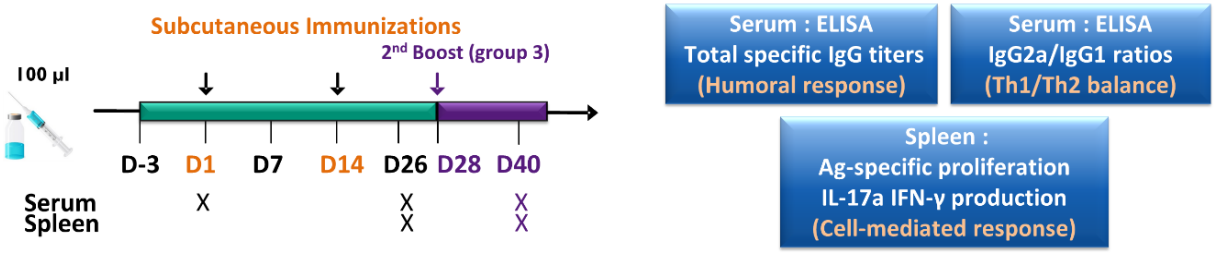

Figure 2.1. Experimental design and timeline of immunizations and sample collection. CD-1 female mice were immunized by subcutaneous injections (100 µl, arrows) two weeks apart at day 1 (D1) and day 14 (D14) as well as at day 28 (D28), for mouse group 3 only. CD-1 mice were divided into 6 groups ($n = 5$ mice per group), see the Materials and Methods section. Group 1 (SCV Inac) received the heat-inactivated *S. aureus* Δ*vraG*Δ*hemB*; group 2 (SCV Live), received the live-attenuated *S. aureus* Δ*vraG*Δ*hemB*; group 3 (SCV Live 3), received the same regimen as group 2, but with an additional boost immunization 2 weeks after the 2nd immunization as illustrated in purple on this timeline; group 4 (SCV Inac + J5), received a combination of the heat-inactivated *S. aureus* Δ*vraG*Δ*hemB* and the heat-inactivated *E. coli* J5; group 5 (SCV Live + J5), received a combination of the live-attenuated *S. aureus* Δ*vraG*Δ*hemB*

and the heat-inactivated *E. coli* J5; and group 6 received only PBS (non-vaccinated control group). The boxes identify the tests performed for serum and spleen samples taken at the indicated time points (X).

2.4.6 ELISAs

Serum total IgG and IgG1/IgG2a isotypes were detected by ELISA against the *S. aureus* Δ*vraG*Δ*hemB* whole cell extract to compare the systemic humoral response generated by the different vaccine versions as previously described [24]. Briefly, Nunc MaxiSorpTM 96-well plates (Thermo Fisher Scientific) were coated with 100 µl of whole *S. aureus* cell extract (10 µg/ml diluted in carbonate/bicarbonate buffer, Sigma) and incubated overnight at room temperature. The plates were then saturated with PBS containing 5% skim milk for 1 h at 37°C, followed by a second blocking step with the addition of 5% porcine serum to prevent unspecific interactions with *S. aureus* protein A and other staphylococcal immunoglobulin binding proteins [24]. One hundred microliters of four-fold serial dilutions of the sera in dilution buffer (PBS with 2% milk, 2% porcine serum and 0.025% Tween 20 [Sigma]) were loaded onto the plates and incubated for 1 h at 37 °C. Plates were then washed three times with PBS containing 0.05% Tween 20, and loaded with 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) previously diluted 1:5000 in the dilution buffer. After 1 h of incubation at 37°C followed by 3 washes, peroxidase activity was detected with the 3,3′,5,5′-tetramethylbenzidine (TMB) reagent at 450 nm using an Epoch microplate reader (Biotek Instruments Inc.) after the addition of 1M H_2SO_4 (KPL Inc., Gaithersburg, MD) according to the manufacturer's recommendations.

2.4.7 Isolation of murine splenocytes

After animals were sacrificed under anesthesia, spleens were harvested aseptically and kept in ice cold Dulbecco's phosphate-buffered saline (DPBS; Wisent). The excised spleens were cut into small pieces and were pressed through 100-µm nylon cell strainers using the plunger end

of a syringe. Cells were then washed with DPBS and centrifuged at 1,800 rpm for 5 min. Cell pellets were suspended in 1 ml of pre-warmed red blood cell lysis solution (Sigma-Aldrich) and incubated for 2 min at 37 °C. After the lysis was stopped by the addition of 30 ml DPBS, cells were centrifuged and suspended in fresh DPBS, and cell count and viability were verified using trypan blue exclusion. Splenocytes were then immediately used for the proliferation and cytokine production assays.

2.4.8 Splenocyte proliferation assay

Freshly isolated splenocytes were used for a proliferation assay in order to measure the specific cellular response of immunized mice after stimulation with inactivated bacteria. Briefly, cells were suspended in complete Roswell Park Memorial Institute (RPMI) medium supplemented with 10 % fetal bovine serum, glutamine, antimycotic-antibiotic solution (1 X of the Penicillin, Streptomycin and Amphotericin B solution from Wisent), non-essential amino acids solution and 2-mercaptoethanol (Sigma-Aldrich). Cell culture reagents were all purchased from Wisent. Cells were adjusted to a concentration of 5×10^5 cells/ml and were distributed in 96-wells culture microplates. They were then stimulated with 5×10^5 CFUs of heat-inactivated *S. aureus* Δ*vraG*Δ*hemB* or *E. coli* J5, 5 µg/ml of Concavalin A (Sigma-Aldrich) or cell culture medium (untreated control). Splenocytes were incubated and proliferation was allowed for 60 h at 37°C in a humidified incubator with 5% CO_2. Cells were then centrifuged, and supernatants were aliquoted and kept at -20°C for cytokine production analysis. Cell pellets were immediately used for the metabolic activity assay, which measures the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) into an insoluble formazan product in metabolically active cells. Briefly, cells were suspended in warm DPBS and 10 µl MTT solution (5 mg/ml) was added before an incubation period of 2 h at 37 °C. Cells were then centrifuged, and an acidic solvent solution of 16 % SDS and 40 % Dimethylformamide, pH 4.7, was added to lyse the cells and solubilize the formazan crystals. The $A_{570\text{nm}}$ of the samples were then measured with a correction at $A_{650\text{ nm}}$ using an Epoch microplate reader (Biotek Instruments Inc.). All assays were

performed in triplicate. Proliferation was then expressed as the ratio of absorbance of treated cells on untreated cells for each mouse-specific splenocytes.

2.4.9 Cytokine production assay

Release of IL-17a and IFN-γ into the supernatant of splenocyte cultures was quantified by a capture enzyme linked immunosorbent assay (ELISA) using DuoSet sandwich ELISA kits (R&D systems, Minneapolis, Mn) and following the manufacturer's recommendations. Peroxidase activity was detected by adding the 3,3′,5,5′-tetramethylbenzidine (TMB) reagent (KPL Inc., Gaithersburg, MD) following the usual procedure of ELISAs.

2.4.10 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (v.6.02). Total IgGs, IgG2a and IgG1 titers as well as splenocyte proliferation ratios were transformed in base 10 logarithm values before being used for statistical analysis. Specific statistical tests used for the analysis of each experiment and statistical significance are specified in the legend of each figure.

2.5 Results

2.5.1 Immunization of mice with live-attenuated SCV stimulates a strong and specific humoral response against *S. aureus* compared to inactivated bacteria.

As the live-attenuated Δ*vraG*Δ*hemB* SCV vaccine has the capacity to elicit strong and specific humoral responses against a variety of mastitis associated *S. aureus* strains without the use of any adjuvants [24], we hypothesized that this high immunogenicity is linked to the live nature of the vaccine. To investigate the impact of this live vaccine composition on both humoral and cell-mediated responses in mice, we compared a group of mice immunized by two injections of

$5x10^7$ CFU the live-attenuated Δ*vraG*Δ*hemB* SCV strain to : (i) a group receiving three injections, (ii) a group immunized with inactivated Δ*vraG*Δ*hemB* SCV bacteria, and (iii) mice receiving a combination of inactivated *E. coli* J5 strain with the live or inactivated SCV (figure 2.1).

Serum total IgGs of immunized mice were assayed in ELISAs for binding to whole cell extracts of *S. aureus*. For every group of mice but one (group 5; live attenuated *S. aureus* combined to inactivated *E. coli* J5), the vaccine subcutaneous injections triggered no adverse effects in mice such as modification of behavior, signs of inflammation or necrosis at the immunization site throughout the immunization period. However, the addition of inactivated J5 bacteria to live SCV in group 5 led to the development of important signs of inflammation in the upper back area near the site of injection in 3 out of 5 mice, 3 to 4 days after the priming injection. The inflammation was sustained for the next few days and was considered too important for the welfare of the animals; thus those 3 mice were euthanized. The two remaining mice developed a moderate inflammation that subsided before the second immunization. The boost immunization did not lead to higher inflammation. Figure 2.2 illustrates the *S. aureus* antigen-specific total IgG titers that were measured in preimmune and immune serum samples of immunized mice. For each vaccination group in which the live vaccine was used (groups 2, 3 and 5), significantly higher IgG titers were detected in the immune samples as compared to their corresponding preimmune sera ($P \leq 0.0001$). Heat-inactivated SCV also led to higher IgG titers ($P = 0.0006$), but these higher immune titers were not observed when inactivated SCV bacteria were combined with inactivated J5 (group 4). Indeed, there was no difference between the preimmune and immune sera for the groups vaccinated with the combination of inactivated SCV and J5 or the PBS control. Conversely, groups that received the live SCV version of the vaccine were all shown to develop statistically higher humoral responses than inactivated formulations or PBS control immunization ($P \leq 0.0001$), independently of the combination with inactivated *E. coli* J5. These results demonstrate that the live vaccine is quite efficient on its own in its ability to raise high antibody titers. These titers are also higher than that obtained with heat-inactivated bacteria. For mice immunized using an additional boost of the live SCV vaccine,

sera yielded a consequential rise of IgG titers (figure 2.2) against *S. aureus* antigens, but this trend was not found to be statistically different to that obtained with the two-dose immunization (P = 0.1270). This suggests that the live strain is already highly immunogenic after two injections.

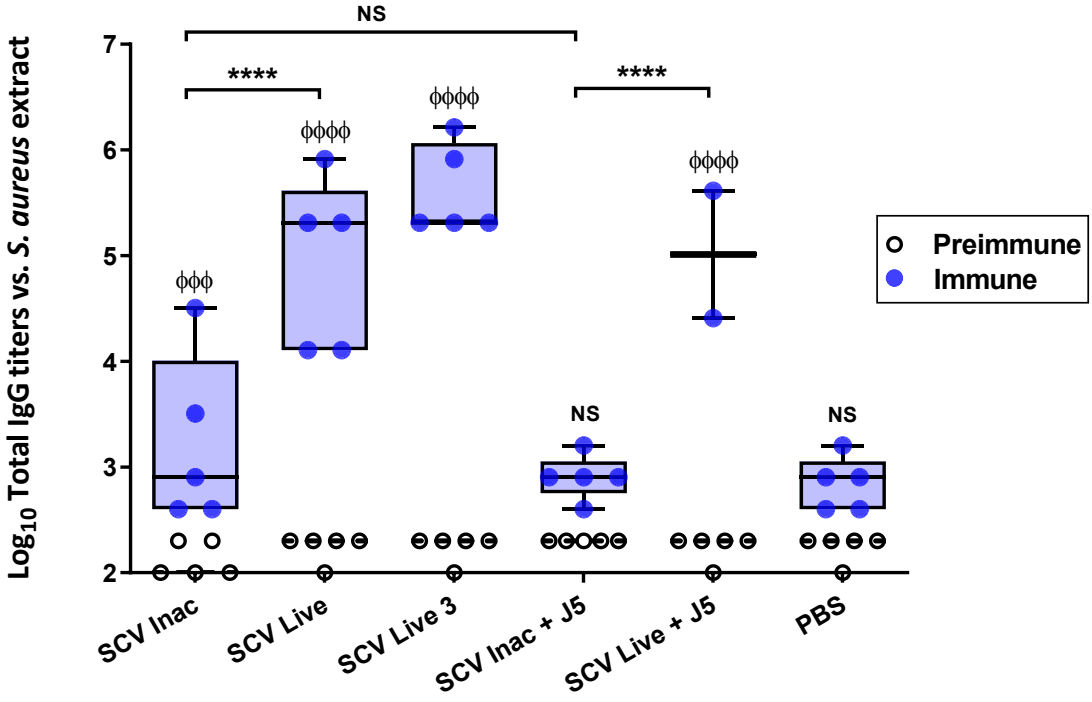

Figure 2.2. Immunization of mice with the live-attenuated double mutant SCV (Δ*vraG*Δ*hemB*) stimulates a strong humoral response against *S. aureus*. The six groups of mice (n = 5 mice per group) are defined in the Materials and Methods section and the immunization schedule is shown in figure 2.1. Sera were collected before priming immunization (preimmune) and 10 days after the boost immunization (immune). Each dot represents the total IgG titer of one mouse against *S. aureus* Δ*vraG*Δ*hemB* whole cell extract, and boxes (upper and lower boundaries) and vertical lines (whiskers) indicate interquartile distances and ranges, respectively. Medians are represented by thick horizontal lines for immune titers and dashed lines for preimmune titers. Immune titers were compared to their corresponding preimmune

titers (Two-way ANOVA and Sidak's multiple comparisons test: φφφφ $P \leq 0.0001$; φφφ $P \leq 0.001$; NS, not significant), as indicated on the top of the corresponding box, or to other groups (Two-way ANOVA and Tukey's multiple comparisons test: ****$P \leq 0.0001$; NS, not significant).

Taken together, these results clearly show that (i) immunization with the Δ*vra*GΔ*hemB* live vaccine can raise a higher humoral response against *S. aureus* antigens than its inactivated counterpart, and that (ii) an additional boost immunization (group 3, three injections in total) yields higher titers that are not significantly different than with two injections (group 2). Furthermore, this humoral response against *S. aureus* (iii) is not enhanced by the combination with *E. coli*. On the contrary, addition of the inactivated J5 to the inactivated SCV vaccine yielded lower titers of *S. aureus* specific IgGs compared to that obtained using the inactivated SCV alone. Also, when combined to the live SCV vaccine, addition of the inactivated J5 resulted in acute inflammatory responses in mice.

2.5.2 Immunizations with the live-attenuated SCV improves the Th1/Th2 immune response balance against *S. aureus*.

In an effort to further characterize the immune response elicited by the various vaccines, the *S. aureus* specific IgG2a and IgG1 isotypes titers were measured as markers for the resulting balance between the Th1 and Th2 responses [29]. Since *S. aureus* has the capacity to invade and survive in non-phagocytic host cells [19] and that antibodies alone are insufficient to protect against this pathogen [3,30], we sought to find out what vaccine formulation could induce a balanced Th1/Th2 type response, hence a higher IgG2a/IgG1 ratio. Figure 2.3A shows that the IgG2a/IgG1 ratio is significantly higher in immune sera from the live-attenuated SCV vaccine group than that obtained by using the inactivated SCV vaccine, suggesting enhanced activation of the cell-mediated immunity pathway in these mice. A lower ratio was also obtained with all the other groups, indicating an excess in IgG1 or equivalent quantities of the two isotypes in the sera of these mice; however, this trend was not found to be statistically significant. Besides, in

inactivated SCV + J5 and PBS groups, very low immune IgG titers are likely the cause of this limited differentiation between one or the other isotype (figure 2.3C). In the same way, addition of the inactivated J5 bacterin to the live-attenuated SCV vaccine (group 5) had no significant effect on the IgG2a/IgG1 ratio when compared with the live vaccine alone. All the mice that received live SCVs produced significantly higher IgG2a titers (figure 2.3B-C). Besides, the apparent reduction in the IgG2a/IgG1 ratio for the live attenuated vaccine that was provided by three injections (figure 2.3A, Live 3), was mainly due to the very high production of IgG1 subsequent to the last boost immunization, and not to lower IgG2a titers as shown in figure 2.3B.

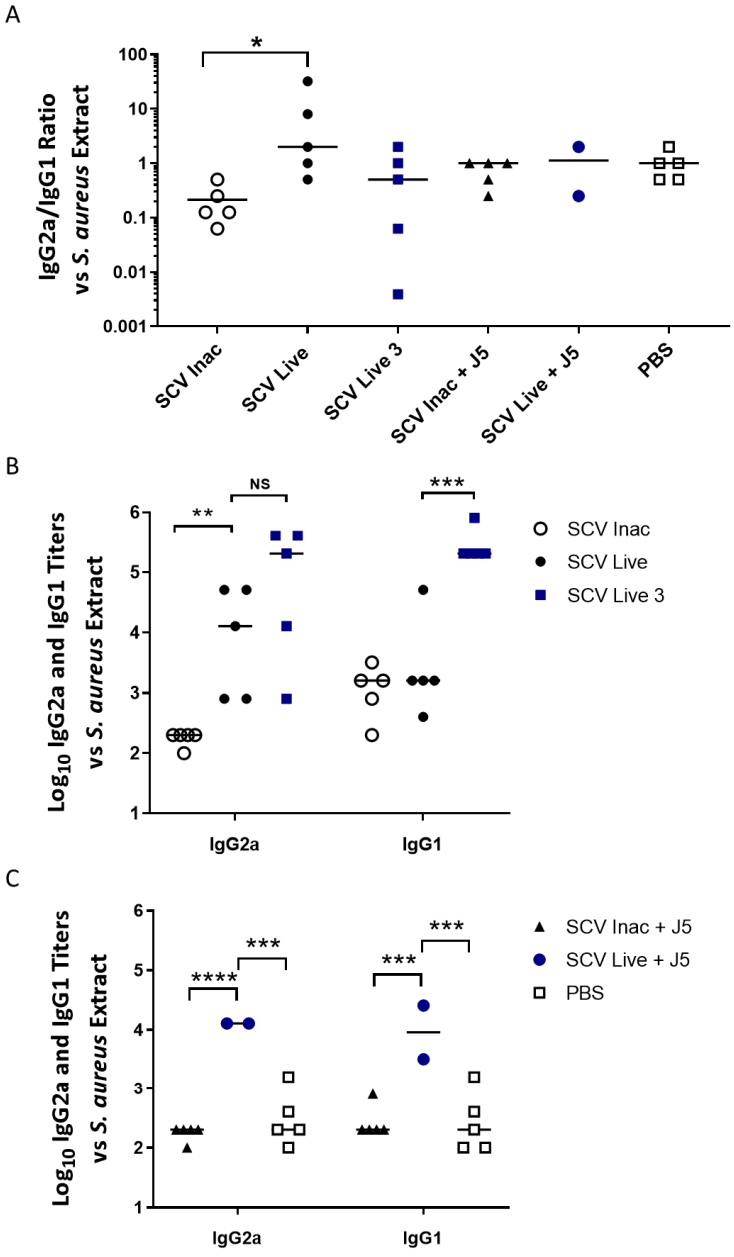

Figure 2.3. Th1/Th2 immune response balance of mice immunized with the live-attenuated double mutant SCV (Δ*vraG*Δ*hemB*) or inactivated bacteria. The six groups of mice shown in (A), (B) and (C) are defined in the Materials and Methods section and the immunization schedule is shown in figure 2.1. Specific IgG2a/IgG1 ratios (A) and IgG2a and IgG1 titers (B-C) of mice against Δ*vraG*Δ*hemB* whole cell extracts. Each dot represents the IgG2a/Ig1 ratio or

the immune IgG2a and IgG1 titer of one mouse. Ratios were calculated using the specific IgG2a/IgG1 titers of each mouse. Medians are represented by horizontal lines. Ratios or titers were compared between each group (A : Kruskal-Wallis test with Dunn's multiple comparison test ; *P ≤ 0.05; B-C : Two-way ANOVA and Sidak's multiple comparisons test; ****P ≤ 0.0001; ***P ≤ 0.001; **P ≤ 0.01; NS, not significant).

2.5.3 Live-attenuated vaccine induces the proliferation of *S. aureus*-specific Th1 and Th17 cell-mediated immunity actors.

In order to evaluate and compare the cell-mediated response elicited by the different vaccine formulations, we collected spleens from sacrificed mice 10 days after the final boost injection. Splenocytes isolated and cultured from vaccinated mice were cultured and assayed for proliferation upon exposure to the inactivated *S. aureus* Δ*vraG*Δ*hemB* and *E. coli* J5. The spleen cells comprise various immunity actors, mainly B and T lymphocytes, but also macrophages, dendritic cells, etc. Proliferation was determined by the ratio of metabolically active cells from every stimulated and unstimulated spleen for each individual mouse. These stimulations were done in triplicate for every mouse and figure 2.4 presents the combined results for all of the mice in one group. Statistical differences arising from these proliferation ratios were calculated by comparing each group of mice to the PBS control group. Mitogenic positive control concanavalin A provoked high proliferation of stimulated splenocytes in every group of mice, as expected (figure 2.4A). Unfortunately, stimulation of splenocytes with inactivated *E. coli* J5 led to unspecific proliferation (figure 2.4B), as seen by the PBS control group being fairly high, indicating possible interactions of the LPS or other molecules from the J5 strain with cell activation. Cells that were stimulated with inactivated *S. aureus* Δ*vra*GΔ*hemB,* however, showed specific proliferation that was well distinguished between the vaccination groups. The Δ*vra*GΔ*hemB* live vaccines (either 2 or 3 doses), led to significantly higher cell proliferation ratios compared to that obtained with the PBS-immunized control (figure 2.4C).

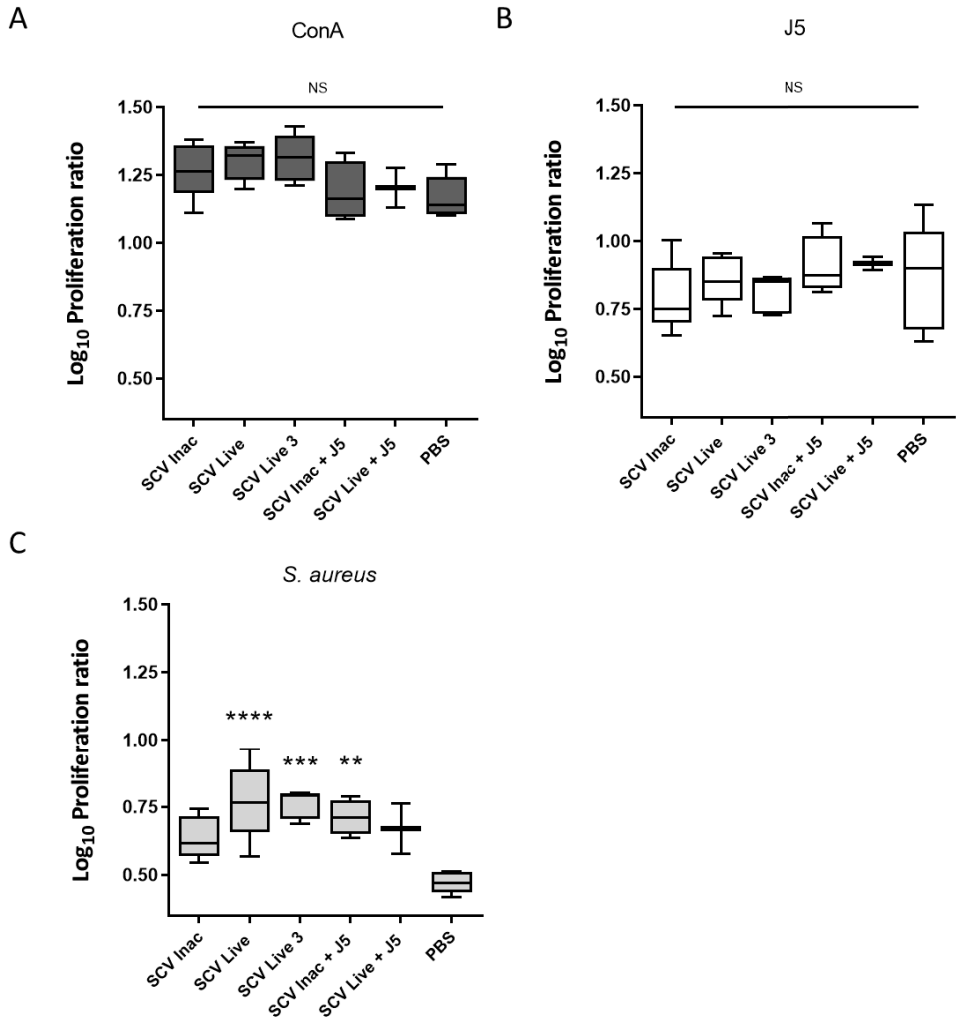

Figure 2.4. *S. aureus*-specific proliferation of splenocytes from vaccinated mice. Cells were stimulated for 60 h with Concanavalin A (A), heat-inactivated *E. coli* J5 bacteria (B) or heat-inactivated *S. aureus* SCV Δ*vraG*Δ*hemB* (C). Each box represents the interquartile distances and ranges of combined results of Log_{10} proliferative ratio of cells isolated from spleens of mice from one group, normalized with unstimulated cells. Horizontal lines represent the medians. Each group of immunized mice were compared to the PBS group (Two-way ANOVA and Tukey's multiple comparisons test: ****P ≤ 0.0001; ***P ≤ 0.001; **P ≤ 0.01; NS, not significant). The six groups of mice shown in (A), (B), and (C) are defined in the Materials and Methods section and the immunization schedule is shown in figure 2.1.

The inactivated *S. aureus* strain combined with the J5 bacterin also generated significant spleen cells proliferation, but to a lower extent. The splenocytes of mice from that group were not found to produce significant amount of Il-17a or IFN-γ in the cell culture media following stimulation (figure 2.5). In fact, only the immunization with the *S. aureus* Δ*vra*GΔ*hemB* live vaccine led to a significant production of IL-17A and IFN-γ by spleen lymphocytes stimulated by heat-inactivated *S. aureus* (figure 2.5). Interestingly, vaccination with the inactivated J5 bacterin combined to the live-attenuated *S. aureus* vaccine slightly suppressed the spleen cells proliferation obtained by using the live vaccine alone (figure 2.4A), despite their equally robust humoral response (figure 2.2). Likewise, cytokine production of splenocytes from this group was also found to be very low compared to the live vaccine.

Overall, these results of proliferation and production of Th17- and Th1-associated cytokines from *S. aureus*-stimulated splenocytes obtained from mice vaccinated with the live-attenuated vaccine provide further evidence of the balanced humoral and cellular immunity responses triggered by the live vaccine.

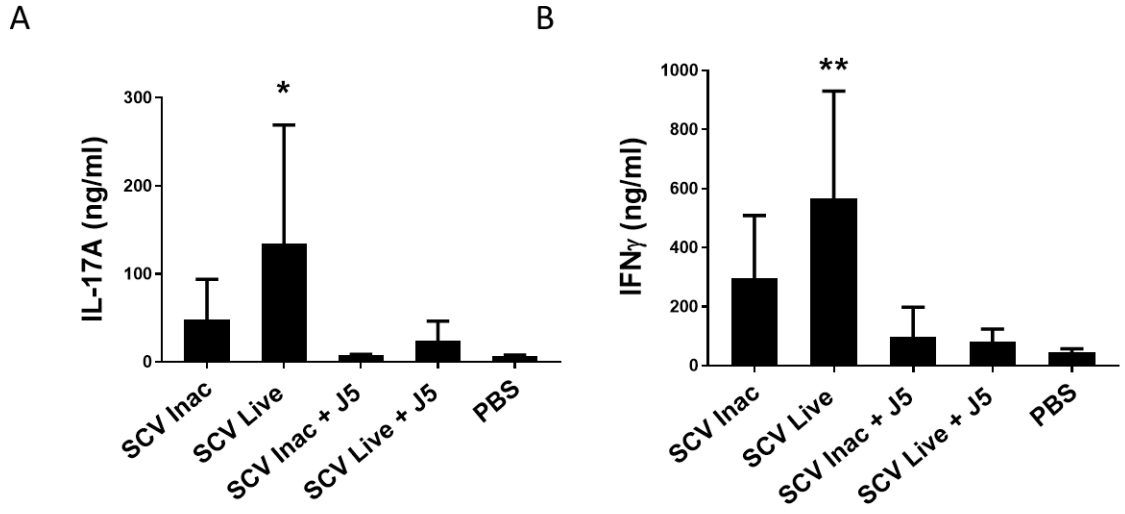

Figure 2.5. *S. aureus*-specific IL-17A and IFN-γ responses elicited by splenocyte proliferation of immunized mice. The six groups of mice shown in (A) and (B) are defined in

the Materials and Methods section and the immunization schedule is shown in figure 2.1. Immunized mice were sacrificed 10 days after the second (boost) immunization, and splenocytes were prepared and stimulated with the heat-inactivated *S. aureus* Δ*vraG*Δ*hemB* SCV for 60 h in RPMI medium. Cells were centrifuged and supernatants were collected. IL-17A (A) and IFN-γ (B) concentrations from cell culture medium quantified by sandwich ELISA and compared to standard curves of recombinant cytokines. Data are expressed as means ± standard errors and statistical differences with the PBS-immunized control mice are shown (Kruskal–Wallis ANOVA with Dunn's multiple comparison test: **P ≤ 0.005; *P ≤ 0.05).

2.6 Discussion

Vaccine development efforts against *S. aureus* suggest that vaccine-induced antibodies may be important but frequently appear insufficient for achieving protection against this pathogen [31]. Currently, as perceived from studies in humans and mice [30,32,33], it is assumed that good Th1 and Th17 responses combined to humoral immunity may be required to obtain at least some efficacy against *S. aureus,* although no specific marker for protection was found thus far [3]. The results of this study support previous observations that live vaccines are often better than their inactivated counterparts at inducing strong and balanced immune responses which could contribute to long-term protection [34]. In order to evaluate the influences of bacterial viability and the combination with a *E. coli* bacterin on the development of a strong and balanced *S. aureus*-specific immune response, we compared the humoral and cell-mediated immunity generated by immunization with several *S. aureus* SCV-based vaccine formulations in mice.

The benefits of the live-attenuated SCV strain were apparent when compared to the same dose of heat-killed bacteria: the live-attenuated vaccine induced higher IgG titers and significantly improved the IgG2a/IgG1 antibody ratio against *S. aureus* in contrast to that observed with the heat-inactivated vaccine. In mice, IgG2a/IgG1 titers ratios are good indicators of the relative importance of the Th1 and Th2 pathways, since these isotypes are produced under the influence of different cytokines during the rise of the acquired immune response. The balanced immune

response triggered in mice that received the live vaccine also provoked a strong proliferation of *ex-vivo* stimulated splenocytes isolated from these animals. These splenocytes were also found to release higher concentration of IL-17 and IFN-γ when compared to every other immunization groups. Despite the fact that no adjuvant was added to the different formulations used and compared in this study, the live SCV vaccine was highly immunogenic by itself, as was formerly observed [24]. This effect was in contrast to the heat-killed formulation, so it is likely that the promotion of higher IgG titers and Th1 and Th17-oriented responses resulted from underlying mechanisms specific to viable organisms.

Vaccines to prevent *S. aureus* infections, in the case of bovine mastitis, have either shown insufficient protection to be accepted for commercialisation or currently only offer limited benefits to be widely used. Whole inactivated bacteria (bacterins) do have the potential to provide antigens that are suitable for raising an immune response, but their protective success is highly dependent on the compatibility and virulence of strains that are present in herds. Moreover, it is apparent that these formulations give rise to immune responses mostly composed of antibody components that can only partly diminish the virulence and/or clinical symptoms of *S. aureus* infections but are not sufficient on their own to prevent colonization [11]. As such, the commercially available StartVac vaccine prepared from killed *S. aureus* and *E. coli* J5 bacterins, aiming at controlling bovine mastitis, has met with contrasting conclusions in recent field trials studies taking a look at its efficacy against *S. aureus* IMIs. Although StartVac had moderate success in reducing the incidence of new *S. aureus* infections [35] or the severity of clinical infections [36], it was also shown to lack protection efficacy against new *S. aureus* IMIs and had no beneficial effect on milk production or survival rates of vaccinated cows in other herds [37]. Differences in herd structure, management and production level, together with differences in *S. aureus* strains type between countries and regions were presented as the probable causing factors for these discrepancies. Here we saw that the combination with J5 bacterin had no beneficial effect or diluted the strength and specificity of humoral and cell-mediated responses against *S. aureus*; in fact, it is likely that the addition of heat-killed *E. coli* may have reoriented the response towards the Th2 pathway, as was seen with the IgG isotypes

ratios and cytokine production assays. Responses to *E. coli* or *S. aureus* IMIs were lately demonstrated to be very contrasting [38,39], at least partly because of the high inflammatory response to LPS found in Gram negative bacteria [40], whereas *S. aureus* can modulate and subverts host responses by suppressing pro-inflammatory pathways [41]. This immunomodulation is quickly followed by the invasion and persistence in host cells, allowing the pathogen to maintain infections for extended periods.

Other ways to improve cell-mediated responses have been experimented in cows. It was shown that antigen-specific Th1 and Th17 inflammatory responses are possible following intramammary immunization of cows with a sensitizing protein [42]. Because of its effect on neutrophil activity, an improved Th17 response could represent an interesting way of enhancing phagocytic activity in the mammary gland, since neutrophils represent the dominant defense in the udder against mastitis-causing pathogens [31]. A recent bovine mastitis vaccination study using intranasal inoculation of cows with purified IsdA and ClfA-cholera toxin A2/B chimeras was attempted in order to stimulate mucosal immunity of the mammary gland [43]. The vaccine induced IL-4 expression but not IFN-γ or IL-17 in peripheral blood mononuclear cells of cows 60 days after the trial. The protection efficacy against *S. aureus* is however still to be determined.

Some adjuvants have the ability to raise balanced and mixed Th1/Th17 responses [30,44] and current research with mice models show interesting candidates using Toll-like receptors (TLRs) agonists, notably TLR-7 which can recognize single-stranded RNAs [45]. In many ways, a bacterial live vaccine can act as an adjuvant by itself because it can stimulate innate immunity in a broad and powerful manner by providing different ligands to these pattern recognition receptors. Interestingly, studies in human have demonstrated that the mechanism behind the high efficacy of live versus killed vaccines resides in the recognition of bacterial viability through the sensing of bacterial RNA by antigen-presenting cells (APCs) TLR8 [46]. APCs then promote differentiation of follicular T helper cells which are essential actors of B-cells activation, affinity maturation and maintenance of humoral memory [47].

Live vaccines are however a source of concern over their safety, thus finding new ways to engineer powerful and stable attenuations without lowering immunogenicity is of great importance for attenuated vaccine development [48]. For this purpose, we previously established the SCV vaccine double mutant strain based on the interruption of gene *vraG*, of importance for oxidative stress and cationic peptide resistance [49–51] and for virulence in bovine IMIs [25], together with the deletion of gene *hemB* which confers a genetically stable SCV phenotype; combined mutations were found to have a large effect on virulence and survival of the parental strain [24]. Moreover, by using a live-attenuated SCV as vaccine, as opposed to a *S. aureus* strain of the normal phenotype, the experimental vaccine from the present study should have the additional advantages of stimulating host cells like if it was infected by both an extra and intracellular pathogen. It is well known that SCVs have their own specific gene expression profile, with highly expressed virulence factors involved in colonization such as adhesins, biofilm production and host cell invasion [14,52,53], whereas the normal phenotype is mostly extracellular and is equipped for dissemination [54]. Since this phenotype switching is a dynamic process that naturally occurs during infections [52,55], increasing the host immune response against the SCV phenotype means that this response could be better suited to recognize the specific *in vivo* antigens exhibited by SCVs during persistence as well as stimulating more efficiently the cellular response. Yet, it was previously demonstrated that the live SCV vaccine shared enough features with the normal phenotype to elicit by immunization of mice a high-titer antibody response able to recognize non-SCV clinical strains from different *S. aureus spa* types [24]. Besides, *in vivo* experiments in mice models do not always translate in cattle, just in the same way as successes of preclinical studies do not always reproduce in clinical trials. Murine models are useful to compare and select multiple vaccine formulations before evaluation of a selected subset in cows. Ovine models [56] might also provide an additional predictive value for efficacy in cows.

On a final note, caution should be used when inducing a robust Th17 response, notably because of its implication in autoimmune diseases, as was previously discussed [3]. However, the development of a vaccine to prevent *S. aureus* infections in humans as opposed to one for bovine

mastitis may overall require different strategies, since *S. aureus* IMIs rarely become life-threatening systemic infections in cows. The subclinical and recurrent aspects of bovine mastitis represent important challenges for milk producers and a vaccine that reduces dissemination during milking, the occurrence of new infections or the duration of IMIs would economically be advantageous, in contrast to a vaccine for humans, which would be required to significantly reduce disease severity or to prevent systemic infections.

In summary, the live-attenuated *S. aureus* SCV vaccine was found to surpass the heat-killed formulation in its ability to raise specific and balanced humoral and cell-mediated responses against *S. aureus*. Besides, addition of the inactivated *E. coli* J5 bacterin to the vaccine offered no benefit regarding the immune response against *S. aureus* as it lowered IgG titers, shifted IgG isotypes towards Th2 response and greatly reduced the production of IFN-γ and IL-17a cytokines from stimulated mouse splenocytes. A strong and balanced immune response is likely the key for handling persistent and recurrent *S. aureus* IMIs. A vaccine based on a live-attenuated SCV could possibly significantly improve protection efficacy against *S. aureus*. A vaccine that could successfully lead to the elimination of *S. aureus* in the early stages of colonization of the udder should bring down transmission rates and eliminate reservoirs of new infections. This would be the key for maintaining and improving long-term competitiveness of milk producers.

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CHAPITRE 3

MOLECULAR BASIS OF THE VIRULENCE ATTENUATION AND ANTIMICROBIAL SUSCEPTIBILITY OF A Δ*VRAG*Δ*HEMB STAPHYLOCOCCUS AUREUS* SMALL-COLONY VARIANT

3.1 Introduction de l'article et contribution des auteurs

La capacité de *S. aureus* à détecter et à s'adapter rapidement à divers stress facilite sa croissance et sa persistance pendant l'infection, et le phénotype SCV de *S. aureus* est connu pour son profil d'expression de gènes qui favorisent sa persistance à long terme chez l'hôte. Après avoir développé et utilisé la souche SCV atténuée Δ*vraG*Δ*hemB* avec succès pour améliorer la vaccination contre *S. aureus*, j'ai voulu investiguer plus en détails quels pourraient être les mécanismes à l'origine de sa forte atténuation. Dans cet article, nous démontrons que le système de régulation GraXRS-VraFG joue un rôle important chez les SCVs, et que la perturbation de VraG entraîne des changements phénotypiques qui diffèrent considérablement des effets de cette mutation dans une souche prototypique non-SCV. De plus, cette étude a pu faire l'analyse du transcriptome de ce SCV atténué et révéler que plusieurs gènes impliqués dans la détection et la résistance aux peptides antimicrobiens cationiques, et certains attributs de sa virulence, étaient affectés par rapport au SCV simple mutant *hemB*. Cette étude a donc permis de mieux comprendre la base moléculaire derrière l'atténuation de la virulence et la sensibilité accrue aux peptides antimicrobiens cationiques et à différents stress *in vivo* du double mutant SCV.

J'ai moi-même, avec l'aide d'Alexis Dubé-Duquette et de Jean-Philippe Langlois, effectué les expériences qui ont mené à cette publication. Jean-François Lucier s'est chargé d'effectuer les méthodes de bio-informatique pour produire les données de transcriptomique et j'ai ensuite pu procéder à leur analyse. J'ai rédigé le manuscrit qui a ensuite été révisé par mon superviseur, Pr. François Malouin. J'ai conceptualisé les expériences avec l'aide du Pr. Malouin.

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Molecular basis of the virulence attenuation and antimicrobial susceptibility of a Δ*vraG*Δ*hemB Staphylococcus aureus* small-colony variant

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3.2 Abstract

The ability of *Staphylococcus aureus* to detect and quickly adapt to various stresses facilitates its growth and persistence during infection. *S. aureus* small-colony variants (SCVs) are known to express a distinct set of genes that allows for long-term persistence in the host. Although phenotype switching from prototypic *S. aureus* to SCV and vice versa is generally viewed as part of the infection process, stable SCVs can be genetically engineered in order to study the characteristics of these persistent strains. We recently described a genetically stable and attenuated SCV mutant strain (Δ*vraG*Δ*hemB*) for use as a live vaccine. Herein, we demonstrate that VraG and the GraXRS regulatory system hold significant roles in the SCV background (*i.e.*, Δ*hemB*), and that disruption of *vraG* in this background results in phenotypic changes that importantly differ from the effects of that mutation in a non-SCV prototypic strain (WT). Notably, cell surface charge and susceptibility to vancomycin and cationic antimicrobials were modified in the Δ*vraG*Δ*hemB* SCV when compared to Δ*hemB* despite no significant difference in cell surface hydrophobicity and membrane potential. Transcriptome analysis revealed that several genes involved in CAMP sensing and resistance and some virulence attributes were differentially expressed in the double mutant following exposure to subinhibitory concentrations of colistin. Interestingly, the *dlt* operon involved in D-alanylation of teichoic acids and the *icaAB* genes involved in biofilm formation were all downregulated in the Δ*vraG*Δ*hemB* SCV strain when compared to Δ*hemB* and such differences were not observed when comparing the non-SCV strains (Δ*vraG vs* WT). Taken together, these findings help to better understand the molecular basis behind the virulence attenuation and the increased susceptibility of the SCV double mutant to cationic antimicrobials.

3.3 Introduction

Staphylococcus aureus is a versatile pathogen with a remarkable ability to adapt to a wide range of host environments and to induce both acute and chronic types of infections in humans and animals. This bacterium uses an abundance of virulence factors, many of which contribute to

host immune response evasion (1, 2), resistance to antibiotics and the ability for long-term persistence in host tissues or cells (3). Such an adaptability also results from its ability to sense and detect the nature of its environment and quickly regulate the expression of virulence factors (4–6). *S. aureus* uses a complex sensorial apparatus involving a variety of two-component systems (TCS) with the capacity to detect various environmental factors and stresses, such as pH, metabolites, oxygen, and antimicrobials (7, 8). In fact, key virulence-associated TCS, such as the quorum-sensing *agr* system (AgrAC), SaeRS and SrrAB, are at the core of the *S. aureus* infection process, which is also complemented by other intracellular regulators of virulence such as proteins of the SarA family and alternative sigma factors like SigB (5). The ability of *S. aureus* to switch from a colonization to a dissemination phase or to manifest an acute or chronic disease inherently depends on the fine tuned balance of these regulators of virulence (9–11).

S. aureus small-colony variants (SCVs) are known to be frequently isolated from chronic infections in human and animals (12, 13) and are associated with therapeutic failures (14, 15) and relapse of disease (16). As their name implies, these variants show a slow growth, leading to the formation of small, non-pigmented and nonhemolytic colonies about one-tenth of the normal *S. aureus* colony size on solid culture medium. The majority of clinical SCVs are thought to derive from specific point mutations that lead to deficiencies in the electron transport chain, altering the oxidative phosphorylation process (14, 17). The low membrane potential of SCVs alters susceptibility to certain antibiotics like aminoglycosides because these drugs need a proton gradient for entering the cells (13, 18), but most importantly, low ATP production and slow growth lead to a distinct gene expression profile allowing long-term persistence in the host. In particular, biofilm production (19, 20), internalization into host cells (21) and resistance to oxidative stress/burst of phagocytes (22, 23) are characteristics that are more prominent in SCVs than that seen in its normal-growth wild-type (WT) counterpart. Because of these characteristics, SCVs are also reported to be more tolerant to several antimicrobials in addition to aminoglycosides, including penicillin and other β-lactams (14), cationic antimicrobial peptides (CAMPs) (24), daptomycin (25) and vancomycin (26).

Phenotype switching from a normal-colony to a SCV phenotype and vice versa is now regarded as a transient but integral part of the infection process and as a survival strategy in harsh environmental conditions (27). Since the SCV phenotype is reversible, genetically stable *S. aureus* SCVs have been widely used for their study, and can be engineered through the deletion of the *hemB* gene (28). In a previous work (29), we have constructed a double mutant that included deletion of *hemB* in addition to the inactivation of gene *vraG,* which was found particularly important for full virulence of *S. aureus* during bovine intramammary infections (IMIs) (30). The gene *vraG* codes for the permease unit of the ABC-transporter VraFG, which is known to contribute together with the GraXRS module as a multicomponent system in CAMPs detection and resistance (31). The Δ*vraG*Δ*hemB* SCV mutant was shown to be greatly attenuated in a bovine mammary epithelial cells invasion/persistence assay and in the murine IMI model (29). Additionally, when used as a live vaccine, high doses of subcutaneous injections of the Δ*vraG*Δ*hemB* SCV mutant could be achieved in mice without provoking any sign of local inflammation or adverse effect while provoking a good immune response (32).

In the present study, we sought to better understand the molecular basis of this virulence attenuation. Since VraG is involved in CAMP detection/repulsion systems and because SCVs show low ATP production and membrane potential, our attention was focused on comparing susceptibility to cationic antimicrobials, cell surface properties and overall gene expression of the parental wild-type *S. aureus* ATCC 29213 strain (WT) and its isogenic single mutants (Δ*vraG* and Δ*hemB*) and double mutant (Δ*vra*GΔ*hemB*).

3.4 Materials and methods

3.4.1 Bacterial strains and growth conditions

Strains used in this study are listed in Table 3.1. *S. aureus* ATCC 29213 and its isogenic mutants Δ*vraG*, Δ*hemB* (SCV) and Δ*vraG*Δ*hemB* (SCV) were previously described (29). The colony morphology of the four isogenic strains is shown in figure 3.1. *S. aureus* strains were grown in

tryptic soy broth (TSB) and agar (TSA) or on brain heart infusion agar (BHIA) (BD, Mississauga, ON, Canada) for SCVs. The construction of the *S. aureus* double mutant strain Δ*vraG*Δ*hemB* was described elsewhere (29).

Table 3.1. *S. aureus* strains used in this study

Strain	Relevant details	Source or Reference
ATCC 29213	Wild-type, normal growth phenotype	ATCC
Δ*vraG*	*vraG* (SACOL0720) intron insertion mutant, isogenic to ATCC 29213, normal growth phenotype	(30)
Δ*hemB*	*hemB*::Em^r; isogenic mutant of ATCC 29213, SCV phenotype	(29)
Δ*vraG*Δ*hemB*	*hemB*::Em^r; isogenic mutant of Δ*vraG,* SCV phenotype	(29)
Mu50	MRSA, VISA, normal growth phenotype	(33)
COL	MRSA, normal growth phenotype	(34)

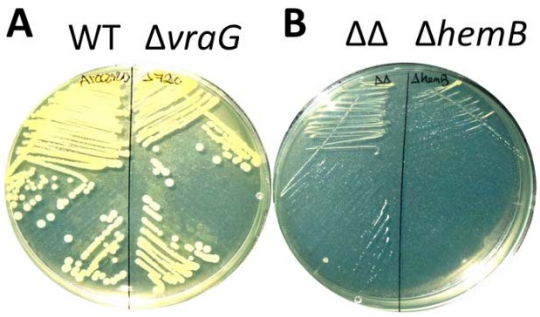

Figure 3.1. Aspects of normal (A) and SCV (B) phenotypes of wild-type (WT) and mutant strains. Strains were grown on TSA plates for 48 h (ΔΔ: Δ*vraG*Δ*hemB*).

3.4.2 Chemical reagents and antibiotics

Tetracycline, vancomycin, gentamicin, colistin, enrofloxacin were obtained from Sigma (Oakville, ON, Canada). Indolicidin cationic peptide (ILPWKWPWWPWRR) was produced by GenScript Inc (Piscataway, NJ). Tetracycline, vancomycin, and gentamicin were solubilized in water at 10 mg/mL.

3.4.3 Antibiotic susceptibility testing

MICs were determined by a broth microdilution technique, following the recommendations of the CLSI (35), although tryptic soy broth (TSB) was the medium used in order to allow sufficient growth of both normal and SCV phenotypes. Also, the incubation period was extended to 48 h for SCVs as described elsewhere (36). Resistance phenotypes were attributed using the following susceptibility breakpoints: tetracycline 16 µg/mL, gentamicin 16 µg/mL and vancomycin 16 µg/mL. Intermediate resistance to gentamicin and vancomycin was defined by concentrations of 8 and 4–8 µg/mL, respectively.

3.4.4 Determination of cell surface charge by the cytochrome c binding assay

The whole cell surface charge was determined by the cytochrome c binding assay according to the method of Meehl *el al*. (37), with some modifications. Briefly, bacterial cultures grown overnight in TSB were pelleted and washed twice with 20 mM morpholinepropanesulfonic acid (MOPS), pH 7. Cell densities were measured spectrophotometrically by absorbance at 650 nm, and a volume of bacterial cells equivalent to 5 optical density units (e.g., 2.5 ml of a culture of A_{650nm} = 2.0) for normal phenotype strains or 10 units for SCVs was pelleted in order to compare equivalent CFUs of bacteria in each sample. Pellets were then resuspended and incubated (with constant agitation) in 500 µl of a solution of 0.5 mg/ml cytochrome c 20 mM MOPS for 10 min at room temperature. The suspension was then centrifuged for 2 min at maximum speed (21 000 *g*), and aliquots of supernatant were collected. The concentration of cytochrome c in the

supernatant fraction was determined by the comparison of its A_{530nm} with a cytochrome c standard curve.

3.4.5 Determination of cell surface hydrophobicity

The microbial adhesion to hydrocarbons assay (38) was used to quantify cell surface hydrophobicity. The protocol described by Lye *et al*. (39) was followed, with few modifications. Briefly, overnight bacterial cultures were harvested and cells were washed in phosphate buffer (50 mM, pH 7.2) followed by suspension in the same buffer to reach an A_{600nm} of 1.0. Then, 3 ml of this suspension was mixed with 0.6 ml of xylene and vortexed for 1 min. The two phases were allowed to separate for 30 min and the aqueous phase was carefully removed to measure the A_{600nm}. Cell surface hydrophobicity (% H) was determined with the following equation:

$$\%H = [(A_0 - A)/A_0] \times 100$$

where A_0 is the initial absorbance (A_{600nm}) of the suspension and A is the final A_{600nm} absorbance of the aqueous phase.

3.4.6 Determination of bacterial cell membrane potential

The relative membrane potential of bacterial cells was measured using the fluorescent dye indicator 3,3′-diethyloxacarbocyanine iodide (DiOC2) (Thermo Fisher Scientific, Ottawa, ON, Canada) as described before (40). Bacteria were grown 24 h at 35°C on BHIA plates and were harvested, washed and suspended in PBS. Then, DiOC2 (3mM) was added and the samples were protected from light and incubated at 35°C with agitation (225 rpm) for 30 min. Using a FACSCalibur (BD, Franklin Lakes, NJ, USA) flow cytometer equipped with a 488 nm laser, cells were analyzed using the red (FL3-H) and green (FL1-H) channels. A higher red on green ratio, or Median Fluorescence Intensity (MFI), represents a higher membrane potential. Data

were analyzed using the FCS Express program. Results were expressed relative to the MFI determined for *S. aureus* ATCC 29213 (WT), representing a MFI of 100%.

3.4.7 Growth conditions and colistin treatment for RNA-seq

Bacterial pre-cultures of the four *S. aureus* isogenic strains (ATCC 29213, Δ*vraG*, Δ*hemB* and Δ*vraG* Δ*hemB*) were grown overnight and diluted in fresh TSB in order to obtain an A_{600nm} of 0.1. Cultures were incubated for 2h at 35°C with orbital shaking (225rpm) to reach the logarithmic growth phase. Cultures were then incubated for an additional 1 h in the same conditions or with the addition of 64 µg/mL of colistin, *i.e.*, a sub-inhibitory concentration for all studied strains. Following treatment of cultures with RNAprotect Bacterial Reagent (Qiagen, Toronto, ON, Canada) according to the manufacturer's recommendations, cells were harvested by centrifugation for 15 min. Bacterial pellets were stored at -80 °C until RNA isolation was performed.

3.4.8 RNA isolation

Bacterial pellets were consecutively warmed and frozen in a 50°C water bath and dry ice for 5 cycles of 10 sec each to weaken the cells prior to treatment with 20 U lysostaphin, 10 U proteinase K and 10,000 U of lysozyme (Sigma). Next, 700 µl RLT buffer from the RNeasy minikit (Qiagen) were added with 0.275 g glass beads (100 µm; Sigma) followed by a 10 min agitation at maximum speed on a vortex platform. Samples were then centrifuged for 30 s at maximum speed to pellet the beads. A volume of 500 µl of ethanol was added to the supernatant, followed by loading on RNeasy minikit (Qiagen) columns. The subsequent purification steps were done according to Qiagen's instructions. DNase digestion using RNase-Free DNase (Qiagen) was performed on-column to clear away residual DNA. RNA samples were eluted in 30 µl RNase-free water and the quality was verified using an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA).

3.4.9 RNA-seq library preparation and sequencing

Total RNA was transformed into TruSeq libraries for sequencing on Illumina NextSeq platform. Briefly, total RNA samples were randomly fragmented, and cDNAs were synthesized. Blunt-ending and phosphorylation were executed followed by dA-tailing. Illumina adaptors were added on the repaired ends. The cDNA coding for rRNA was removed using the Evrogen duplex-specific nuclease (Cedarlane, Burlington, ON, Canada). The quality and the quantity of the library was evaluated using an Agilent 2100 bioanalyzer (Agilent) and Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Burlington, ON, CA) by following the manufacturer's instructions. Sequencing data was obtained from paired-end reads (2 × 75 bp) using the generated 10nM RNA-seq library.

3.4.10 RNA-seq data analysis

Transcriptome reads were analysed with the Genpipes RnaSeq pipeline (https://bitbucket.org/mugqic/genpipes/src/master/pipelines/rnaseq/). Briefly, reads were trimmed using Trimmomatic software (41) and then aligned against the reference genome of ATCC 29213 (GCA_001267715.2) using STAR (42). RNASeq data expression was then analysed using the Tuxedo tools (cufflinks, cuffmerge, cuffquant, cuffdiff, cuffnorm) (43). Differential expression was then analysed using DESEQ (44) and EDGER (45). Differential expression results were then imported to a custom SQLite database (https://www.sqlite.org/index.html). In parallel, since genome annotation of ATCC 29213 is not quite well present in downstream analysis tools, we did a gene-by-gene comparison between genome annotation ATCC 29213 and NCTC 8325 using the BLAST (46) where the best hit in NCTC 8325 was kept for each ATCC 29213 gene. These mapping results were imported to the SQLite database. Finally, custom queries were built to retrieve differentially expressed genes (DEGs) with a $\log_2$ Fold Change ($\log_2$FC) expression of ≤ -1.5 or ≥ 1.5 between each isogenic strains pair and in presence or absence of colistin. Kyoto encyclopedia of genes and genomes

(KEGG) pathway annotation of DEGs was performed by KOBAS (47) based on KEGG database (48).

3.4.11 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (v.8.3.1). Specific statistical tests used for the analysis of each experiment and statistical significance are specified in the legend of each figure.

3.5 Results

3.5.1 *vraG* has a greater influence on CAMP susceptibility in the SCV background.

In order to increase our understanding of the molecular basis of the virulence attenuation of the Δ*vraG*Δ*hemB* strain, the susceptibility of this double mutant to different antimicrobials was evaluated and compared with that of the single mutants Δ*vraG* and Δ*hemB* and that of the parental strain ATCC 29213 (figure 3.2). Tetracycline or enrofloxacin, demonstrated similar MIC values across the panel of strains despite any of the mutation and whether the strain showed the normal growth phenotype (*i.e.*, *S. aureus* ATCC 29213 and Δ*vraG*) or not (SCV Δ*vraG*Δ*hemB* and SCV Δ*hemB*). In addition, since the defective electron transport chain of the SCVs affects the electrochemical gradient across the bacterial membrane and reduces the penetration of aminoglycosides (14), the MIC for gentamicin was found to be at least 16 times higher for the *hemB* mutants (Δ*hemB* and Δ*vraG*Δ*hemB*) than that recorded for their respective normal phenotype strains (ATCC 29213 and Δ*vraG*, respectively), as usually predicted for respiratory-impaired SCVs (13). Strain Δ*hemB* also showed a reduced susceptibility to vancomycin (a 4 to 8-fold increase in MIC *vs* ATCC 29213), thereby giving it an intermediate clinical resistance phenotype. This vancomycin resistance was not observed in the double mutant Δ*vraG*Δ*hemB* even though it has the SCV phenotype. However, the Δ*vraG*Δ*hemB* mutant showed an important increase in susceptibility to indolicidin (32 to 64-fold) versus

Δ*hemB*. Additionally, colistin, another CAMP which is usually not effective against Gram positive bacteria, showed activity (MIC of 128 µg/ml) against the Δ*vraG*Δ*hemB* strain despite that no significant activity was observed against the other strains. A difference of at least 8-fold was observed between the two SCVs since the *hemB* mutant showed no inhibition of growth at the highest tested concentration of colistin, *i.e.,* 1024 µg/ml. Together these results indicate that the increased susceptibility of *vraG* mutants to cationic antimicrobials and vancomycin is greater in the SCV background compared to that seen in the normal-growth phenotype strains.

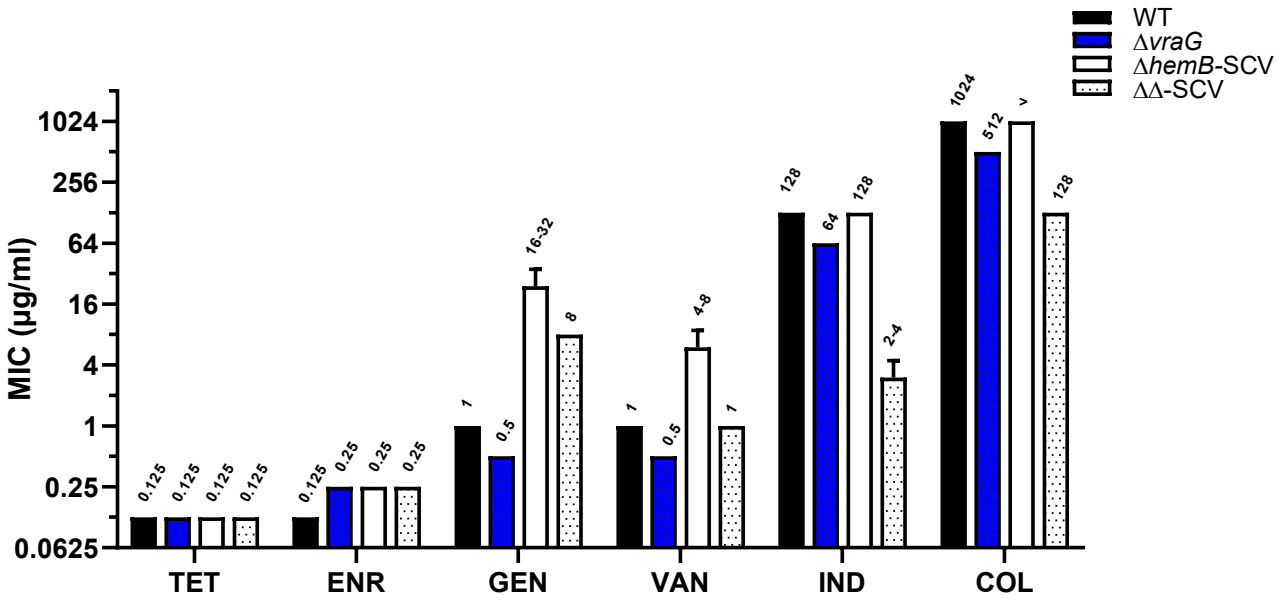

Figure 3.2. Susceptibility profile of *S. aureus* ATCC 29213 and isogenic mutants to antimicrobials. Bars represent minimal inhibitory concentration (µg/ml) of different antimicrobials against ATCC 29213 (WT) and its isogenic mutants. ATCC 29213 and Δ*vraG* are normal-growth phenotype strains, whereas Δ*hemB* and Δ*vraG*Δ*hemB* (ΔΔ) are SCVs. TET, tetracycline; ENR, enrofloxacin; GEN, gentamicin; VAN, vancomycin; IND, indolicidin; COL, colistin. The symbol ">" indicates that the MIC is higher than 1024 µg/ml (the highest concentration tested).

3.5.2 *vraG* plays a role in the net bacterial cell surface charge of SCVs

Since the most striking differences in antibiotic susceptibility tests were obtained using cationic antimicrobials, we attempted to determine if the mutations in the double or single mutants were directly altering the cell surface charge, and if such differences were also more noticeable in the SCV phenotype. It was previously demonstrated that mutations in the *graR* response regulator and the *vraG* permease genes could have a significant effect on the net surface charge, which could be easily evaluated by a cytochrome c binding assay (37). This effect is thought to be the direct consequence of the expression of the *dlt* operon (under the control of the GraXRS-VraFG system), which is crucial for the incorporation of D-alanine residues into the negatively charged cell-wall lipoteichoic acid (LTA), and consequently renders bacteria less susceptible to cationic molecules (49). After the incubation for 10 min with cytochrome c, the capacity of the WT strain ATCC 29213 to bind this highly positively charged protein was compared to the isogenic mutants (figure 3.3). Control strains with known cell wall alterations were also used for comparison, i.e., the vancomycin-intermediate resistance strain Mu50 and the MRSA strain COL. As illustrated in figure 3.3, no significant difference was found between ATCC 29213 (WT) and the two other reference strains in their ability to bind cytochrome c. As expected, cytochrome c binding was affected by the single *vraG* mutation and the *vraG* mutant presented a more negative net surface charge (i.e., more cytochrome c binding) than that measured for the WT, though the significance was low ($P = 0.0468$). Interestingly, the SCV *hemB* mutant had significantly less binding of cytochrome c when compared with the WT ($P=0.0001$) while addition of the *vraG* mutation to the SCV background (strain Δ*vraG*Δ*hemB*) showed the most negatively charged surface of all strains. The extent of cytochrome c binding was thus inversely correlated to the extent of susceptibility to colistin and indolicidin. In order of cytochrome c binding, we thus have Δ*hemB* < WT < Δ*vraG* < Δ*vraG*Δ*hemB*, the latter (*i.e.*, the most negatively charged) being the most susceptible to cationic antimicrobials. Hence, the effect of the mutation in *vraG* on the net surface charge was much greater in the *hemB* SCV background than that observed in the normal-growth phenotype background.

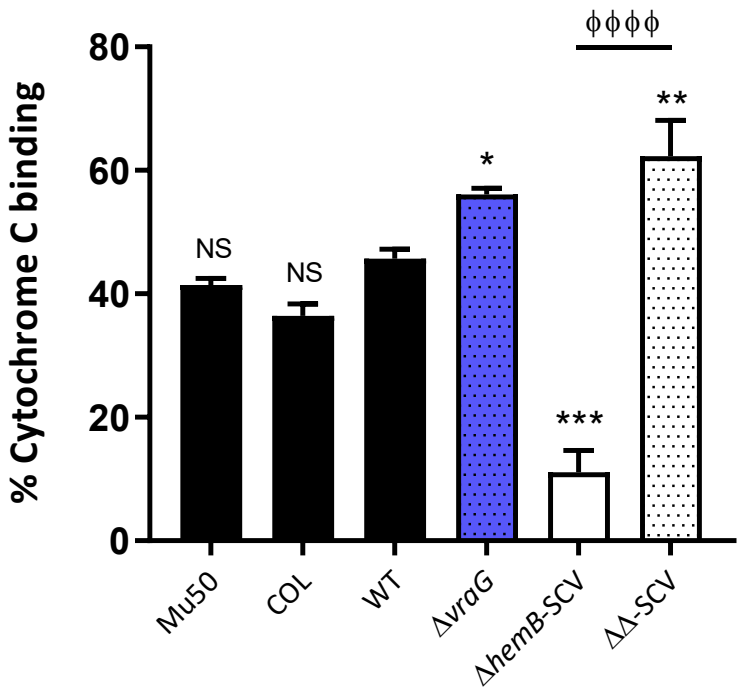

Figure 3.3. Binding of cytochrome c to *S. aureus* whole cells. The results are reported as percent of cytochrome c biding after a 10 min incubation with *S. aureus* cells at room temperature. Detailed below the graph are the reference strains (Mu50 and COL) and ATCC 29213 (WT), and the genotypes of the WT isogenic mutants Δ*vraG*, Δ*hemB*, and Δ*vraG*Δ*hemB* (ΔΔ). Strains with the SCV phenotype are indicated. Data represent the means and standard deviations from three independent experiments done in duplicate. Statistical significances with the "*" symbols are compared to the WT, and that with the "Φ" symbols is for the comparison between the SCV strains (One-way ANOVA and Dunnett's multiple comparisons tests: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ΦΦΦΦ, $P \leq 0.0001$; NS, no significant difference).

3.5.3 The high cell surface hydrophobicity of SCVs is independent of *vraG*

Other cell-surface characteristics of bacteria, such as lipid composition and cell hydrophobicity, are known to alter susceptibility to certain antibiotics (50, 51). Having demonstrated that the net

surface charge of the SCV Δ*vraG*Δ*hemB* was considerably more negative than that of the SCV Δ*hemB* (figure 3.3), we sought to determine if other cell surface differences could be determined among the strains. Hence, we used the well-established microbial adhesion to hydrocarbons (MATH) test (38) to measure and compare cell surface hydrophobicity. In this assay, WT *S. aureus* ATCC 29213 was found to have a relatively low hydrophobicity (figure 3.4), like previously described for that strain and other strains of the normal-growth phenotype (40). When the WT was compared to the Δ*vraG* strain, no significant difference in hydrophobicity was observed, in contrast to the results obtained in the cell surface charge assay. However, hydrophobicity values for both SCV mutants were found to be considerably higher than that of the WT strain with mean percentages of 82.7% for Δ*hemB* and of 73.5% for Δ*vraG*Δ*hemB* ($P = 0.0002$ and $P = 0.0005$, respectively). There was also a difference between the Δ*vraG* and Δ*vraG*Δ*hemB* but this difference was a little less pronounced ($P = 0.0019$). Additionally, as indicated in figure 3.4, there was no significant difference found between the SCV strains, suggesting that disruption of gene *vraG* had no measurable impact on hydrophobicity, both in the SCV or in the normal-growth phenotype background. Together, these findings indicate that SCVs have a significantly higher cell surface hydrophobicity than strains of the normal-growth phenotype, and that the effects of *vraG* mutation observed on the net surface charge (figure 3.3) does not correlate with cell surface hydrophobicity.

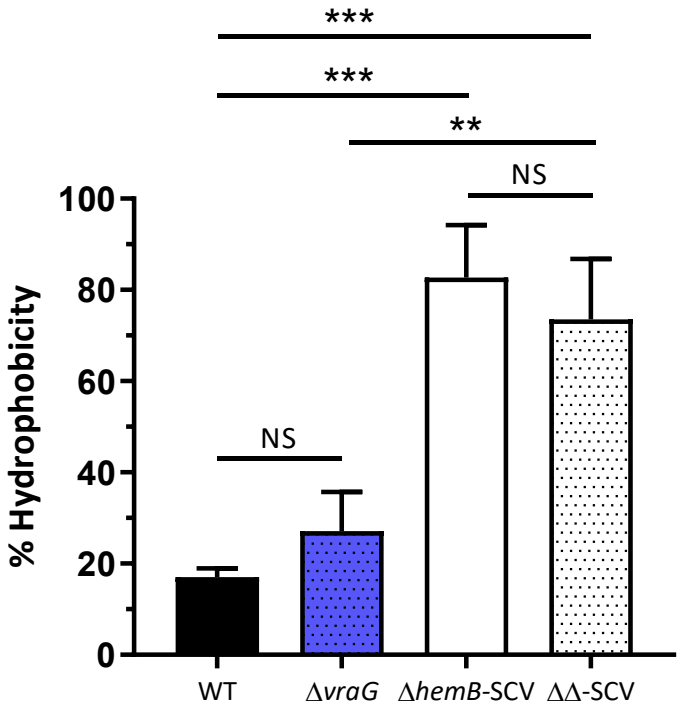

Figure 3.4. Cell surface hydrophobicity of *S. aureus* ATCC 29213 and isogenic mutants. The results are reported as percent of hydrophobicity (proportion of bacterial cells transferred to xylene in a PBS-xylene emulsion). Strains are listed below the graph, with the reference strain ATCC 29213 (WT) and the genotypes of the WT isogenic mutants Δ*vraG*, Δ*hemB*, and Δ*vraG*Δ*hemB* (ΔΔ). Strains with the SCV phenotype are indicated. Data represent the means and standard deviations from three independent experiments done in duplicate. Statistical significance between the indicated strains is shown (One-way ANOVA and Tukeys's multiple comparisons test: **, $P \leq 0.01$; ***, $P \leq 0.001$; NS, no significant difference).

3.5.4 The reduced membrane potential of SCVs is independent of *vraG*

Genetically stable SCVs of *S. aureus* have been studied for several years and many phenotypic effects resulting from the disruption of the electron transport chain by deletion of the gene *hemB* have been documented (14). For instance, the relatively low membrane potential of SCVs is

consequent to the *hemB* mutation and is a characteristic that we have previously reported for that strain as well as for another *hemB* mutant (40). In order to determine if *vraG* also influences the membrane potential, we compared the membrane potential of the WT strain to our series of isogenic mutants. As we expected from previous observations (40), the deletion of *hemB* effectively reduced the membrane potential to the very low value of 10.2% relative to the WT (figure 3.5). The Δ*vraG*Δ*hemB* SCV also had a significant decrease of its membrane potential compared to the WT, but the membrane potential of the double mutant did not differ from that of the Δ*hemB* SCV. Besides, although the membrane potential was slightly reduced in the *vraG* mutant compared to that of the WT strain, this reduction was not found to be statistically significant. Hence, the low membrane potential of SCV strains (figure 3.5) inversely correlates with the high cell surface hydrophobicity of these strains (figure 3.4) and VraG does not contribute to these features. Together, these results suggest that although deletion of gene *hemB* strongly decreases the membrane potential, a mutation in *vraG* has no supplemental effect on the membrane potential, either in the SCV or in the normal-growth phenotype background.

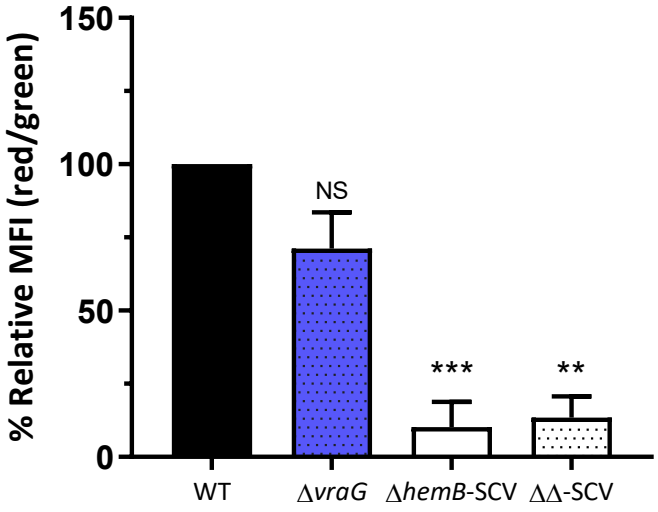

Figure 3.5. Membrane potential (MFI) of *S. aureus* ATCC 29213 and isogenic mutants. The results are reported as percent of MFI relative to the ATCC 29213 strain (WT). Strains are

listed below the graph, with the reference strain ATCC 29213 (WT) and the genotypes of the WT isogenic mutants Δ*vraG*, Δ*hemB*, and Δ*vraG*Δ*hemB* (ΔΔ). Strains with the SCV phenotype are indicated. Data represent the means and standard deviations from two independent experiments done in triplicate. Statistical significance is related to the WT (One-way ANOVA and Dunnett's multiple comparisons test: **, $P \leq 0.01$; ***, $P \leq 0.001$; NS, no significant difference).

3.5.5 Transcriptomic analysis: General features

To gain further insight into the effects of the *vraG* gene disruption in the SCV background, its effect on CAMP susceptibility and to better understand the molecular basis of the double mutant attenuation *in vivo*, RNA-seq analyses were performed on *S. aureus* ATCC 29213 and its isogenic mutants in presence or absence of a sub-inhibitory concentration of colistin. The cDNA libraries of the 8 *S. aureus* samples were constructed, sequenced, and generated with a total of 21,550,152 to 31,812,198 reads which were mapped to the reference genome of *S. aureus* ATCC 29213 (GCA_001267715.2). Gene expression levels determined as read counts or reads per kilobase per million (RPKM) showed a total of 2,804 genes that were expressed in the colistin-induced and control samples.

3.5.6 Differential gene expression of the isogenic strains in presence or absence of colistin

DEGs were retrieved from comparisons between 8 selected pairs of samples (without colistin: Δ*vraG*Δ*hemB vs* Δ*hemB;* Δ*hemB vs* WT*;* Δ*vraG vs* WT*;* Δ*vraG*Δ*hemB vs* Δ*vraG.* The same comparisons were made with the samples from strains exposed to colistin). Differentially expressed ORFs were cross-referenced with the *S. aureus* NCTC 8325 genome, and functionally annotated. The numbers of up- or down-regulated genes specific to the presence or absence of colistin, or common to both conditions, are shown in figure 3.6 for each pair of strains. DEGs with a $\log_2$FC expression of ≤ -1.5 or ≥ 1.5 in presence or absence of colistin and gene predicted functions are detailed for each comparison and are listed in supplementary data sets S1, S2, S3

and S4. KEGG pathway enrichment analysis was also performed for the SCV DEGs (with and without colistin) from the Δ*vraG*Δ*hemB vs* Δ*hemB* comparison (supplementary data set S5). More specifically, the KEGG pathways "*Staphylococcus aureus* infection", "Cationic antimicrobial peptide resistance", "ABC transporter", "Valine, leucine and isoleucine biosynthesis" "Biosynthesis of amino acids" "2-Oxocarboxylic acid metabolism" "D-Alanine metabolism" and "Quorum sensing" were significantly enriched (Table 3.2; see complete analysis results in supplementary data set S5).

Table 3.2. Significantly enriched KEGG pathways of differentially expressed genes in Δ*vraG*Δ*hemB* compared to Δ*hemB*.

KEGG Term	KEGG ID	Input No. of genes	Background No. of genes	P-Value
Staphylococcus aureus infection	sao05150	17	37	1.46E-06
Cationic antimicrobial peptide (CAMP) resistance	sao01503	7	12	0.000717837
ABC transporters	sao02010	18	86	0.003232507
Valine, leucine and isoleucine biosynthesis	sao00290	6	13	0.004274246
Biosynthesis of amino acids	sao01230	18	101	0.013497155
2-Oxocarboxylic acid metabolism	sao01210	6	20	0.021591138
D-Alanine metabolism	sao00473	3	6	0.037160584
Quorum sensing	sao02024	11	60	0.041410585

Hence, expression of genes linked to virulence, stress response, antimicrobial sensing/resistance and cell wall synthesis and turnover were examined in more detail. A summary of key differential gene expression between the Δ*hemB* SCV and the WT, and between the double mutant and each of the single mutants is illustrated in figure 3.7. Genes obtained from enriched KEGG pathways in the SCV double mutant were more specifically identified. Expression of *vraG* and the *dlt* operon was found to be reduced in the double mutant when compared to the Δ*hemB* SCV, both in presence and absence of colistin. The biofilm-associated genes *icaAB* (part of the *icaADBC* operon and responsible for poly-N-acetylglucosamine (PNAG) synthesis) were also less expressed in the double mutant upon exposition to colistin, whereas these genes were more expressed in the SCV strains compared to that observed in their normal-colony strains under normal conditions. Gene *fosB*, encoding a bacillithiol-S-transferase and associated with fosfomycin and oxidative stress resistance (52), was also found to be downregulated in the SCV double mutant in presence of colistin. Also, expression of *essB*, encoding a membrane component of the ESAT-6-like secretion system (ESS) (53), was lower in the double mutant than that seen in the Δ*hemB* strain in absence of colistin, although the ESS *esxA* and *esxB* effectors seemed to be more expressed in this comparison. Lastly, gene *chp,* coding for the

chemotaxis inhibitory protein of *S. aureus* (CHIPS), was also downregulated in the double mutant Δ*vraG*Δ*hemB* when compared to Δ*hemB*. Expression of known gene modulations was also verified using the WT *vs* Δ*hemB* and Δ*vraG vs* WT comparisons, such as the "downregulation" of gene *hemB* in the *hemB* mutant (figure 3.7) and other genes related to the SCV phenotype expression profile. For instance, genes known to be associated with the invasive virulence of the normal phenotype such as *hla*, *sak*, *hlgB*, *hlgC* and others (supplementary data S2) were downregulated in Δ*hemB* in both conditions. These virulence genes were also found to be downregulated in the double mutant when compared to the *vraG* mutant (supplementary data S4).

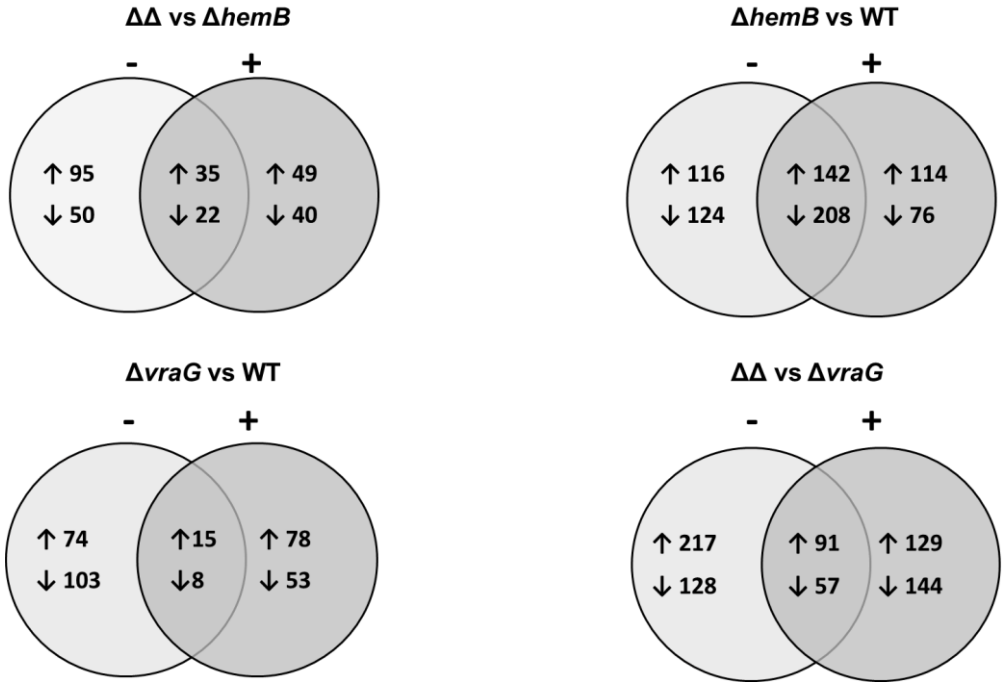

Figure 3.6. Number of differentially expressed genes among isogenic strains in presence or absence of colistin. The reference strain ATCC 29213 (WT) and the WT isogenic mutants Δ*vraG*, Δ*hemB*, and Δ*vraG*Δ*hemB* (ΔΔ) were compared in pairs in the presence or absence of colistin, and a second comparison was made to retrieve DEGs that are common or specific to the absence and/or presence of colistin. Venn diagrams show number of functionally annotated

genes that were at least 1.5 Log_2FC up- or downregulated (symbolized as ↑ and ↓ arrows, respectively) in the indicated pairs of strains in absence (–, light grey) or presence (+, darker grey) of a sub-inhibitory concentration of colistin (64 µg/ml).

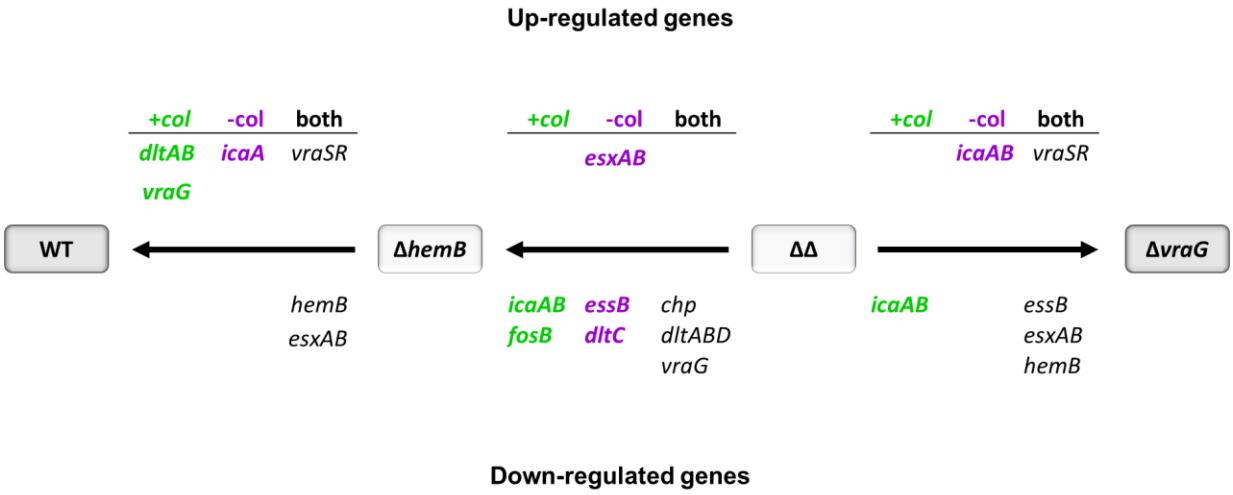

Figure 3.7. Summary of key differentially expressed genes in presence or absence of colistin. Up- and down-regulated ($Log_2FC > 1.5$ or < -1.5) genes selected from the total DEGs from figure 3.6 are illustrated. The reference strain ATCC 29213 (WT) and the WT isogenic mutants Δ*vraG*, Δ*hemB*, and Δ*vraG*Δ*hemB* (ΔΔ) are compared in pairs following the direction of the arrow. For example, gene *icaAB* is downregulated in ΔΔ compared to Δ*hemB* and also when compared to Δ*vraG*, while absence of gene *hemB* is only apparent in Δ*hemB* compared to WT or in ΔΔ compared to Δ*vraG*. Strains with the SCV phenotype are shown in white boxes whereas strains with the normal-growth phenotype are in light-grey boxes. DEGs in presence or absence of colistin are represented in bold green and purple, respectively, and DEGs that were revealed in both conditions are in black.

3.6 Discussion

The aim of this study was to determine the basis behind the strong *in vivo* attenuation of a *S. aureus* Δ*vraG*Δ*hemB* mutant (29) and to investigate the hypothesis that SCVs display a higher resistance to certain cationic antimicrobials at least partly via the influence of the GraXRS-VraFG CAMP repulsion system. With this in mind, we measured antimicrobial susceptibility, investigated cell surface properties and compared gene expression profiles of *S. aureus* ATCC 29213 and the three isogenic mutants to discern the effects of the SCV phenotype and the *vraG* mutation.

The MICs of tested antimicrobials for the single *hemB* and *vraG* mutants followed a pattern in concordance with what was expected from previous investigations, notably for gentamicin and vancomycin (13, 54). The *hemB* mutant showed a reduced susceptibility to vancomycin, a phenomenon that had already been observed in Δ*hemB* SCVs, although the reduced efficacy of vancomycin was not always visible through a change in MICs (26, 55). Since the MIC of vancomycin dropped back to the level seen against the parental strain when testing the Δ*vraG*Δ*hemB* strain, we suspected a particular influence of the *vraG* mutation in the SCV background. VraG was previously shown to be involved in intermediate resistance to vancomycin (37) and other CAMPs through its interaction with GraXRS (31, 56, 57). We did not however observe a significant difference in the vancomycin MIC for the *vraG* mutant compared to the WT (only 2-fold), whereas a *vraG* mutant of the VISA Mu50 strain had previously shown a MIC reduction by a factor of 4 compared to the parent (37). Indeed, transcriptional studies had previously linked the overexpression of *graRS* in strains with intermediate resistance to vancomycin (58). This may indicate a more subtle basal expression of *graXRS-vraFG* in VSSA strains such as ATCC 29213, however this should be confirmed with other strains. Besides, the most striking difference observed in MICs was the significant increase in susceptibility of the Δ*vraG*Δ*hemB* SCV to indolicidin and colistin compared to the *hemB* single mutant, while the effect on the susceptibility to these cationic antimicrobials in the normal-growth phenotype Δ*vraG* strain relative to the WT was very modest. Again, this showed

that VraG plays a significant role in managing the effects of vancomycin and cationic antimicrobials in the SCV background.

Some of the cell surface properties reported for the four isogenic strains also helped to further discriminate and better explain the specific effects of the *hemB* and *vraG* mutations on antimicrobial susceptibility. As demonstrated before (40), the low membrane potential of SCVs inversely correlates with the high cell surface hydrophobicity of these strains, but we showed here no influence of VraG in both of those cell surface properties. Our findings demonstrate that despite the low membrane potential recorded for both SCVs, Δ*hemB* and Δ*vraG*Δ*hemB*, the latter displayed a much higher susceptibility to vancomycin, colistin and indolicidin. This contrasts with the general suggestion that the low susceptibility of respiratory-deficient SCVs to some CAMPs is directly due to the alteration of membrane potential (14, 24). In fact, we found a better correlation between the cell surface charge and susceptibility to cationic antimicrobials. Indeed, the cytochrome c binding assay showed that for either type of strains (SCV or normal-growth phenotype strains), the level of susceptibility to cationic antimicrobials depended on the relative cell surface charge. In *S. aureus*, a key mechanism used to confer cationic antimicrobial resistance is via the incorporation of positively charged residues into the cell surface to generate electrostatic repulsion of CAMPs (59). The VraFG module takes part in CAMPs sensing and interacts with GraXRS to act as a multi-component system (MCS) that regulates its own expression (*vraFG*) together with *dltABCD* and *mprF*, involved in D-alanylation of cell wall teichoic and lipoteichoic acids and lysinylation of membrane phosphatidylglycerol, respectively (31, 57). Our transcriptomics studies following colistin treatment revealed that operon *dlt* and *vraFG* were up-regulated in presence of colistin in the Δ*hemB* SCV relative to the WT, but down-regulated in both conditions in the Δ*vraG*Δ*hemB* SCV when compared to Δ*hemB* (figure 3.7). These genes did not appear to be differentially expressed when comparing Δ*vraG* and WT, and raw data analysis revealed that these genes were in fact only slightly expressed in both of these normal-growth phenotype strains and in both assay conditions (presence or absence of colistin). In fact, we could not find differential expression of *vraG* between Δ*vraG* and WT strains. Considering the Δ*vraG* strain was obtained

by knockout of *vraG* via the insertion of a group II intron, as opposed to complete deletion as with Δ*hemB*, it is likely that the disrupted gene product was expressed, sequenced and mapped, although being non-functional. Hence, since VraFG regulates its own expression through the detection of CAMPs, its higher basal expression in the Δ*hemB* SCV (compared to the WT) may have been sufficient to detect colistin and upregulate *vraFG* and *dlt*, but this upregulation was impaired in Δ*vraG*Δ*hemB*. In contrast, it is possible that the duration of treatment or concentration of colistin selected (1/2 of the MIC of Δ*vraG*Δ*hemB*, *i.e.*, only 1/16 of the MIC for WT) were not ideal to stimulate expression of those genes in the normal-colony background.

The *dlt* operon is an important effector of *S. aureus* resistance to CAMPs and neutrophil killing (56, 60) and it was recently shown that the GraXRS-VraFG MCS is also used by *S. aureus* to sense and adapt to the acidified phagolysosome of macrophages (61), allowing its intracellular growth and persistence in this rather toxic environment. The high virulence attenuation (29) and relative hypersusceptibility of the double mutant to cationic antimicrobials we observed here seem to support these findings. Interestingly, several genes associated with cell-wall synthesis and stress response were found to be up-regulated in both SCVs strains when compared to their corresponding normal phenotype strain, such as *murA*, *murF*, *ddl* and the cell-wall stress regulator system *vraSR,* as reported before (62). This could indicate that the disruption of *vraG,* although increasing susceptibility of the double mutant to cationic antimicrobials, may not affect the expression of other cell-wall stress response effectors that seem to be normally upregulated in the SCV phenotype (62). Of note, increased resistance to vancomycin was associated with a higher expression of GraXRS-vraFG and/or VraSR and their regulon in VISA strains (63).

Other genes involved in the virulence of *S. aureus* were differentially expressed in the Δ*vraG*Δ*hemB* strain. Notably, figure 3.7 shows that the biofilm-associated *ica* operon was more expressed in the double mutant compared to the Δ*vraG* strain, as would be expected in the comparison of a SCV and a normal-growth strain (11). However, the expression of the *ica* operon was markedly diminished under colistin treatment in the double mutant compared to the Δ*hemB* strain (figure 3.7). The *icaADBC* operon codes for products conducting the synthesis

and accumulation of poly-N-acetyl-glucosamine (PNAG; also known as PIA, polysaccharide intercellular adhesin) on the cell surface (64). Hence, the reduced expression of *ica* under colistin stress might indicate that biofilm production is impaired in the double mutant in this condition, at least compared to Δ*hemB*. This may have an impact *in vivo* where host cationic peptides can be found, and it will have to be confirmed experimentally. Biofilms both protect bacteria from host defenses and antibiotics (65) and could yet be another protecting factor that is lost in the double mutant and thus explaining its low virulence *in vivo*. In the same way, the susceptibility to fosfomycin and response to H_2O_2 of the Δ*vraG*Δ*hemB* strain should be examined as its down-regulation of *fosB* compared to that seen in the Δ*hemB* strain in the presence of colistin (figure 3.7) might also indicate an increased sensitivity of the double mutant to oxidative stresses (52). SCVs are known to be more resistant to oxidative stress and to the oxidative burst of neutrophils (22, 23) and a weakening of the double mutant's ability to detect, respond and resist to these stresses could contribute to the understanding of its low survival in the *in vivo* environment (29). Interestingly, expression of the gene *chp*, coding for the chemotaxis inhibitory protein of *S. aureus* (CHIPS), was reduced in the double mutant, compared to the respective Δ*hemB* isogenic strain (figure 3.7). CHIPS is involved in the reduction of neutrophil chemotaxis by blocking the Formyl Peptide Receptor (FPR) and C5a Receptor on neutrophils (66). This small excreted protein is produced during the early growth stages to rapidly block essential steps of the early inflammatory response (67). However, this protein is highly human specific (66), therefore its implications in animal models is hard to verify. Finally, the ESAT-6 secretion system (*essB* gene) and secreted effectors (*esxAB*) are less expressed in the double mutant compared to the Δ*vraG* strain. In the absence of colistin, the double mutant also expresses less transporter (*essB*) than the single Δ*hemB* mutant although the effectors seem to be more expressed (figure 3.7). This is intriguing because *essB* is required for secretion of the effectors and a mutation in *essB* has been shown to impair *S. aureus* resistance to neutrophil killing and virulence in murine infection models (53, 68). It was however shown that *esxA* is expressed by itself as a single transcript and is regulated in a balanced manner by opposing roles of sigma factor B and sigma factor B-controlled SpoVG (69). Esx effectors are believed to have a role in the modulation of intracellular persistence (70) and also affect the

immune response of dendritic cells (71). This is interesting because the reduced expression of *essB* in the double mutant corroborates our observation that the double mutant is less fit to persist within host cells (29). Evidently, these Ess differences in expression observed between the double mutant and the single *hemB* mutant will have to be confirmed by other methods like qPCR and could be investigated to demonstrate *in vitro* or *in vivo* significance.

In summary, this study further supports the assertion that *vraG* is an important gene required for virulence and resistance to CAMPS and host defenses and demonstrates that it plays a particularly important role in the SCV phenotype. The slow growth phenotype, the high relative negative cell surface charge, the high susceptibility to cationic antimicrobials and the low expression of the *dlt* and *ica* operons involved in surface charge modification and biofilm formation, respectively, could all explain to some extent the significant attenuation of the Δ*vraG*Δ*hemB* SCV demonstrated *in vivo* (29). This further supports the possibility of using this double mutant as a safe live-attenuated vaccine in veterinary medicine (32).

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3.9 Supplemental material

Dataset S1. List of differentially expressed genes in Δ*vraG*Δ*hemB* compared to Δ*hemB* in presence (+) or absence (-) of colistin.

Dataset S1.xlsx

Dataset S2. List of differentially expressed genes in Δ*hemB* compared to WT in presence (+) or absence (-) of colistin.

Dataset S2.xlsx

Dataset S3. List of differentially expressed genes in Δ*vraG* compared to WT in presence (+) or absence (-) of colistin.

Dataset S3.xlsx

Dataset S4. List of differentially expressed genes in Δ*vraG*Δ*hemB* compared to Δ*vraG* in presence (+) or absence (-) of colistin.

Dataset S4.xlsx

Dataset S5. KEGG pathway enrichment analysis of differentially expressed genes in Δ*vraG*Δ*hemB* compared to Δ*hemB* (colistin + and -).

Dataset S5.xlsx

CHAPITRE 4

RÉSULTATS COMPLÉMENTAIRES

4.1 Contexte des résultats complémentaires

Conjointement aux investigations visant à vérifier les principales hypothèses de recherche de ce doctorat, certaines expériences complémentaires non présentées dans les articles composant les **Chapitres 2 et 3** de ce document ont eu une place importante parmi les travaux académiques effectués tout au long de ces études. Celles-ci ont pu de façon parallèle répondre à des objectifs de recherche et grandement contribuer à soutenir la démonstration de la pertinence de l'utilisation de la souche SCV atténuée en vaccination. Ces résultats ont entre autres permis la bonification et complétion d'un article (Côté-Gravel et al., 2016) (présenté en **Annexe II**), établissant l'atténuation de la souche SCV Δ*vraG*Δ*hemB,* avec la démonstration de son immunogénicité chez la souris. D'autres expériences en vaccination effectuées lors de ces études mettent en valeur les avantages de l'utilisation des protéines de fusion et de la combinaison de la souche SCV atténuée à des protéines sous-unitaires pour l'amélioration de la réponse immune contre des antigènes de *S. aureus*. Les résultats de celles-ci ont pu être intégrées comme soutien à une demande de brevet maintenant publiée (**Annexe III**). Ces résultats complémentaires sont ainsi abordés dans le présent chapitre.

4.2 Objectif spécifique 1 : Démonstration de la stimulation de la réponse humorale chez la souris par le vaccin SCV atténué

Suite à la publication de mon mémoire de maîtrise en 2015, le manuscrit faisant office de partie centrale de cet ouvrage fut hautement modifié et bonifié lors des études doctorales pour devenir enfin l'article final publié en 2016 (Côté-Gravel et al., 2016; voir **Annexe II**; https://doi.org/10.1371/journal.pone.0166621). Les principales expériences supplémentaires

qui n'étaient pas alors comprises dans ce mémoire ont consisté en la démonstration et l'évaluation de la réponse humorale générée par la vaccination de souris avec la souche vivante atténuée SCV, constituant l'objectif spécifique 1 de ce doctorat. Pour cette expérience, des souris ont été immunisées selon le plan expérimental résumé et illustré à la figure 4.1. En bref, les souris CD-1 ont été réparties au hasard en 4 groupes: le groupe 1 (N=3) a reçu une dose de 10^6 UFC de bactéries atténuées Δ*vraG*Δ*hemB*; le groupe 2 (N=3), 10^7 UFC et le groupe 3 (N=3), 10^8 UFC de bactéries resuspendues à la concentration voulue dans 100 µl de PBS. Cette formulation de vaccin vivant atténué a également été comparée à un vaccin sous-unitaire utilisant uniquement la protéine staphylococcique IsdH purifiée comme antigène. La protéine recombinante IsdH de *S. aureus* a été produite dans *E. coli* tel que précédemment décrit (Ster et al., 2010); le 4^e groupe de souris (N=6) a donc été immunisé par deux injections sous-cutanées dans le cou, à trois semaines d'intervalle, en utilisant 20 µg de protéine IsdH combiné à l'adjuvant EMULSIGEN®-D (25% v/v) dans un volume de 100 µl. Les souris ont par la suite été immunisées par deux injections sous-cutanées dans le cou des trois formulations de vaccin atténué ou du vaccin recombinant, à deux semaines d'intervalle. Des échantillons de sang pour les sérums ont été prélevés juste avant la première injection (sérums pré-immuns) et de 10 à 21 jours après l'immunisation de rappel (sérums immuns). La description détaillée des méthodes de préparation de la culture pour le vaccin vivant, des extraits bruts bactériens et de la détection des IgGs par ELISA sont abordés en totalité en **Annexe II** (Côté-Gravel et al., 2016).

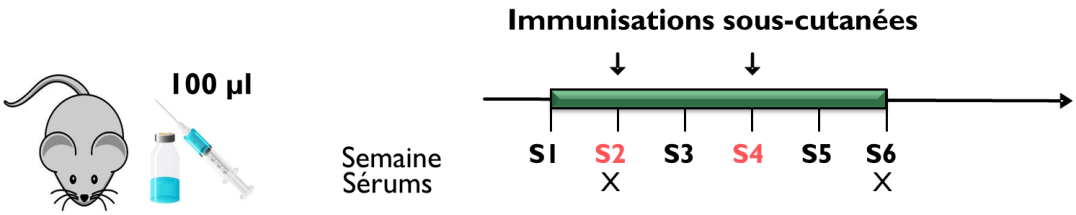


Figure 4.1. Plan expérimental de la démonstration de la stimulation de la réponse humorale par la souche atténuée Δ*vraG*Δ*hemB* (Côté-Gravel et al., 2016).

Il est intéressant de relever que suite à l'immunisation sous-cutanée des souris par la souche vivante atténuée, et ce même à des doses aussi élevées que 10^8 UFC, aucun effet secondaire indésirable n'a pu être relevé tout au long de la période d'immunisation. De plus, les quantités croissantes du double mutant vivant Δ*vraG*Δ*hemB* ont pu éliciter la production de titres croissants d'anticorps IgG systémiques dirigés contre un extrait de cellules entières de la souche vaccinale (figure 4.2A). Les titres des sérums immuns étaient en effet significativement plus élevés que ceux des sérums pré-immuns pour tous les groupes ayant reçu le vaccin vivant, démontrant la spécificité de la réponse contre *S. aureus*.

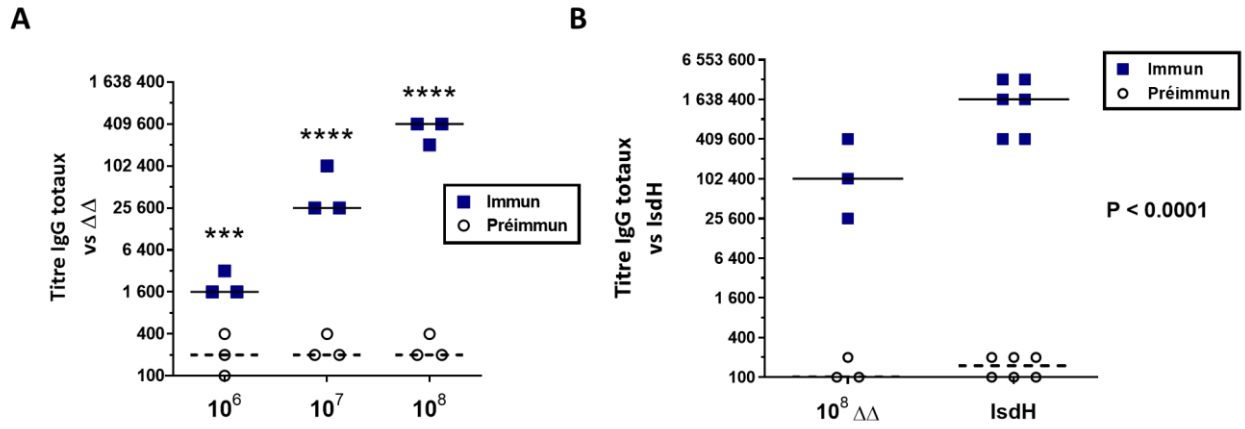


Figure 4.2. Réponse humorale spécifique de souris immunisées avec la souche vivante atténuée Δ*vraG*Δ*hemB* ou la protéine recombinante IsdH de *S. aureus*. (A) Titres sériques d'IgG totaux de souris immunisées avec des doses croissantes (10^6, 10^7 et 10^8 UFC) de la souche vivante atténuée Δ*vraG*Δ*hemB* (ΔΔ) dirigés contre l'extrait brut de cellules bactériennes de la souche ΔΔ. (B) Titres sériques d'IgG totaux dirigés contre la protéine recombinante IsdH de souris immunisées avec 10^8 UFC de la souche Δ*vraG*Δ*hemB* (10^8 ΔΔ) ou avec la protéine IsdH recombinante (IsdH). Chaque symbole représente le titre total d'IgG d'une souris. Les titres immuns (carrés bleus) ont été comparés à leurs titres pré-immuns (cercles vides) correspondants, et les lignes horizontales représentent les médianes (***: $P \leq 0.001$; ****: $P \leq 0.0001$) (Côté-Gravel et al., 2016).

Puisque la dose de 10^8 est parvenue à éliciter d'une quantité relativement élevée d'IgG spécifiques contre *S. aureus* sans pour autant produire d'effets néfastes chez la souris, cette formulation fut directement comparée à celle du vaccin sous-unitaire IsdH pour sa capacité à générer des anticorps pouvant reconnaître cette protéine de surface régulée par le fer. Il a pu en effet être possible de générer des titres relativement élevés d'IgG spécifiques contre IsdH en immunisant avec la souche vivante atténuée (figure 4.2B). Afin de déterminer si la balance des réponses Th1/Th2 étaient affectées par la quantité de bactéries atténuées ou le type de formulation, les titres d'isotypes IgG2a (marqueur de la réponse Th1) et IgG1 (marqueur de la réponse Th2) furent également mesurés. Fait intéressant, la dose de 10^7 UFC a pu générer des ratios d'IgG2a/IgG1 plus élevés que la dose 10^8 (figure 4.3A) ; il semblerait que cette diminution du ratio lors de l'augmentation de la dose à 10^8 UFC soit expliquée par un « boost » additionnel de la production d'IgG1 plutôt qu'une diminution des titres en IgG2a (figure 4.3C). De plus, la formulation de 10^7 UFC de vaccin atténué a pu être montrée comme plus balancée vers la réponse Th1 que celle du vaccin sous-unitaire, comme le montre son ratio IgG2a/IgG1 significativement plus élevé contre l'antigène IsdH (figure 4.3B).

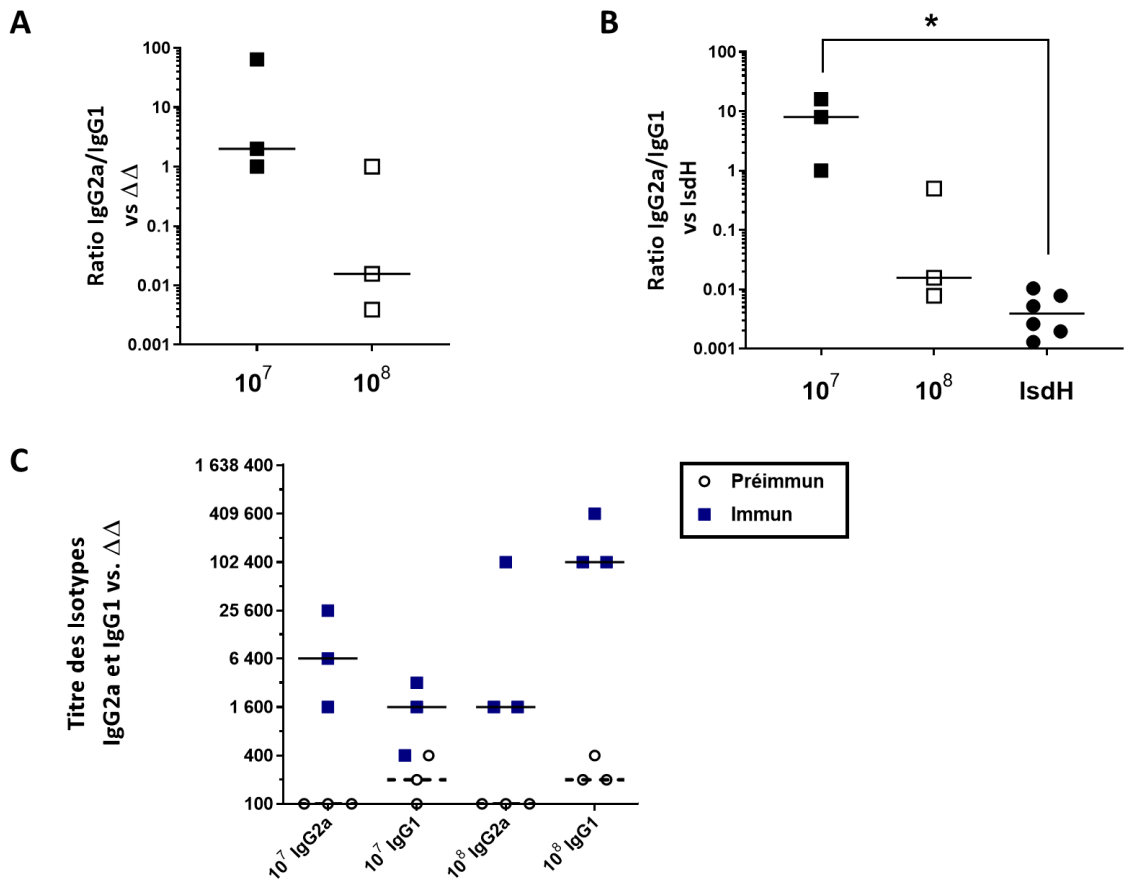


Figure 4.3. Balance Th1/Th2 de la réponse humorale spécifique de souris immunisées avec la souche vivante atténuée Δ*vraG*Δ*hemB* ou la protéine recombinante IsdH de *S. aureus*. Ratios des isotypes IgG2a/IgG1 dirigés contre (A) l'extrait brut de cellules bactériennes de la souche Δ*vraG*Δ*hemB* (ΔΔ) ou contre (B) la protéine IsdH de souris immunisées avec 10^7 UFC ou 10^8 UFC ou la protéine IsdH recombinante. (C) Titres sériques des isotypes IgG2a et IgG1 dirigés contre l'extrait brut de cellules bactériennes de la souche ΔΔ de souris immunisées avec 10^7 UFC ou 10^8 UFC de la souche ΔΔ. Chaque symbole représente le ratio IgG2a/IgG1 (A-B) ou le titre total d'IgG (C) d'une souris, et les lignes horizontales représentent la médiane. La significativité statistique entre les groupes comparés est montrée (*: $P \leq 0.05$) (Côté-Gravel et al., 2016).

Finalement, cette réponse humorale spécifique à *S. aureus* a été testée dans sa capacité à détecter des souches d'intérêt clinique dans le contexte de la mammite bovine. Pour ce faire, des extraits bruts de souches de *S. aureus* de type clonal *spa* répandus dans les troupeaux laitiers canadiens (Pichette-Jolette et al., 2019) ont été préparés et utilisés comme antigènes dans les plaques ELISA. La réponse humorale suivant l'augmentation de la quantité de bactéries utilisées pour immuniser les souris a pu effectivement générer des titres croissants aussi contre les différentes souches testées dans les ELISAs (figure 4.4 ; ici seulement la dose de 10^8 est présentée), montrant que les anticorps générés contre la souche vaccinale sont capables de détecter des composants bactériens partagés chez les *S. aureus*.

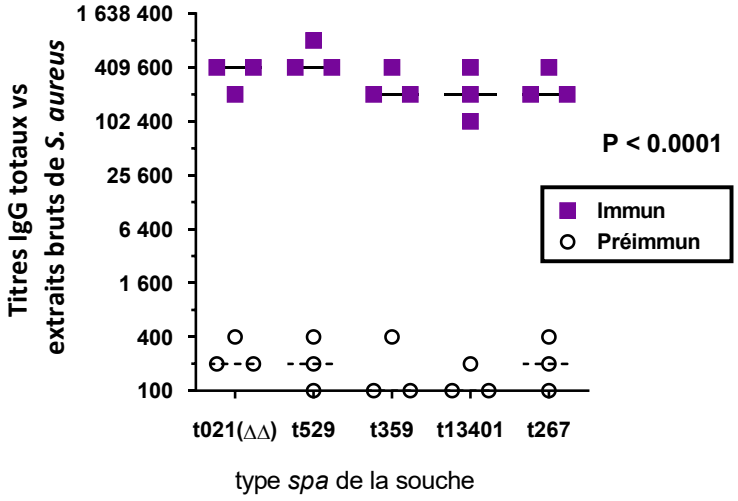


Figure 4.4. Réponse humorale spécifique de souris immunisées avec la souche vivante atténuée Δ*vraG*Δ*hemB* contre des isolats cliniques de mammite bovine. L'immunisation avec la souche vivante atténuée Δ*vraG*Δ*hemB* (ΔΔ) confère des titres d'IgG élevés contre des composants partagés par des souches de mammite de types *spa* communément retrouvés dans les troupeaux laitiers canadiens. Chaque symbole représente le titre d'IgG totaux d'une souris immunisée avec 10^8 UFC de ΔΔ contre l'extrait bactérien entier de la souche du type *spa* indiqué. Les médianes sont représentées par des lignes continues pour les titres immuns et des lignes pointillées pour les titres pré-immuns. Tous les titres immuns ont été comparés à leurs titres pré-immuns correspondants ($P < 0.0001$ pour tous les groupes) (Côté-Gravel et al., 2016).

Ces résultats ont donc pu clairement démontrer que l'immunisation avec la souche vivante atténuée Δ*vraG*Δ*hemB* peut effectivement déclencher une réponse humorale forte, balancée et spécifique contre *S. aureus*. En outre, les résultats montrent que cette souche SCV partage suffisamment de caractéristiques communes avec les souches de mammite bovine pour que la réponse en anticorps permette également la reconnaissance de souches d'intérêt clinique et d'origines clonales diverses.

4.3 Démonstration des avantages de la vaccination par des protéines de fusion sous-unitaires sur la réponse humorale chez la souris

Cette expérience a été effectuée dans le cadre de ces études doctorales dans le but de soutenir la demande de brevet WO2018072031 présenté en **Annexe III**. Les propriétés immunogènes de différentes protéines recombinantes de *S. aureus* (SACOL0029, SACOL1867 et une fusion de SACOL0029 et SACOL1867), issues des gènes précédemment montrés comme importants pour la virulence de *S. aureus* (Allard et al., 2013), ont été évaluées chez la souris. Afin de comparer les réponses immunitaires obtenues pour ces antigènes, soit sous forme monovalente (SACOL0029 ou SACOL1867 uniquement) ou une forme multivalente (SACOL0029-1867 en fusion, et SACOL0029 + SACOL1867 en combinaison), des souris recevant une quantité équimolaire exacte de ces protéines sous ces formes monovalentes ou multivalentes ont été comparées, tel qu'illustré par la figure 4.5. Plus précisément, vingt souris femelles CD-1 ont été réparties au hasard dans 4 groupes: le groupe 1 (N=5) a reçu 5 µg de la protéine de fusion SACOL0029-1867 (Fusion 29-67); le groupe 2 (N=5) a reçu 1.15 µg de SACOL0029 et 3.69 µg de SACOL0029-1867 (combinaison); le groupe 3 (N=5) a reçu 1.15 µg de SACOL0029 (Ag-29) et le groupe 4 (N=5) a reçu 3.69 µg de SACOL1867 (Ag-67). Pour la préparation des immunisations, les protéines ont été mélangées et mises en suspension dans du PBS pour obtenir la quantité équimolaire de chaque antigène de la fusion dans un volume final de 100 µl.

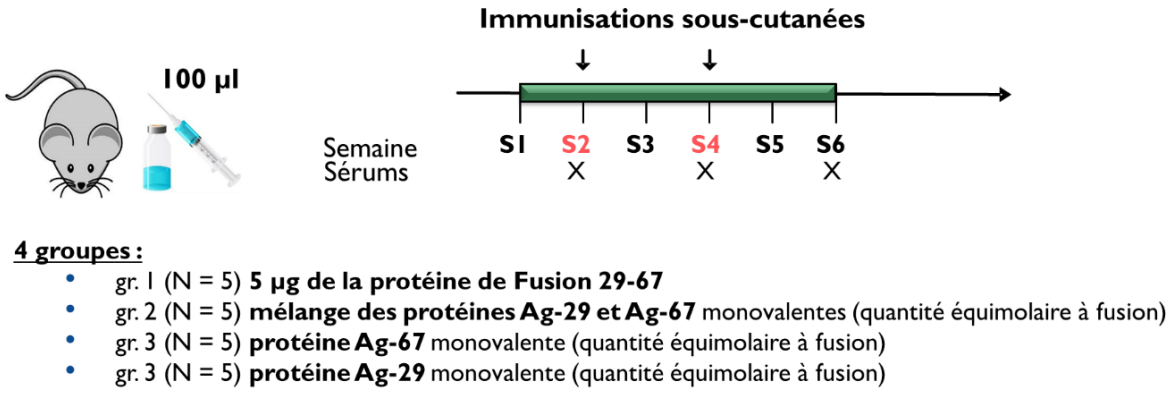


Figure 4.5. Plan expérimental de la démonstration de l'amélioration de la réponse humorale spécifique par la fusion de protéines importantes pour la virulence de *S. aureus*.

Les antigènes recombinants ont été conçus et produits par GenScript, Inc. (Piscataway, NJ) selon la méthode détaillée plus précisément dans la demande de brevet (**Annexe III**). Les souris ont ensuite été immunisées par deux injections sous-cutanées dans le cou à deux semaines d'intervalle et les prélèvements sanguins pour les sérums et la détection des IgGs par ELISA ont été effectués suivant la méthode précédemment décrite dans la section 4.2.

Dans cet essai, l'antigène Ag-67, correspondant à la protéine SACOL1867 sous forme monovalente, fut utilisé pour tapisser le fond des plaques ELISA pour comparer les titres IgGs entre les groupes de souris. La figure 4.6 montre que, lorsqu'inclus dans un polypeptide de fusion, cet antigène (Ag-67) peut induire une réponse humorale forte et spécifique significativement plus élevée que celle obtenue avec une forme monovalente de cet antigène ou avec une combinaison multivalente des deux parties polypeptidiques de la fusion. On constate ainsi qu'en plus de de fournir des réponses immunitaires contre de multiples cibles polypeptidiques, ces antigènes fusionnés fournissent un avantage supplémentaire au vaccin en améliorant considérablement leurs titres d'anticorps respectifs.

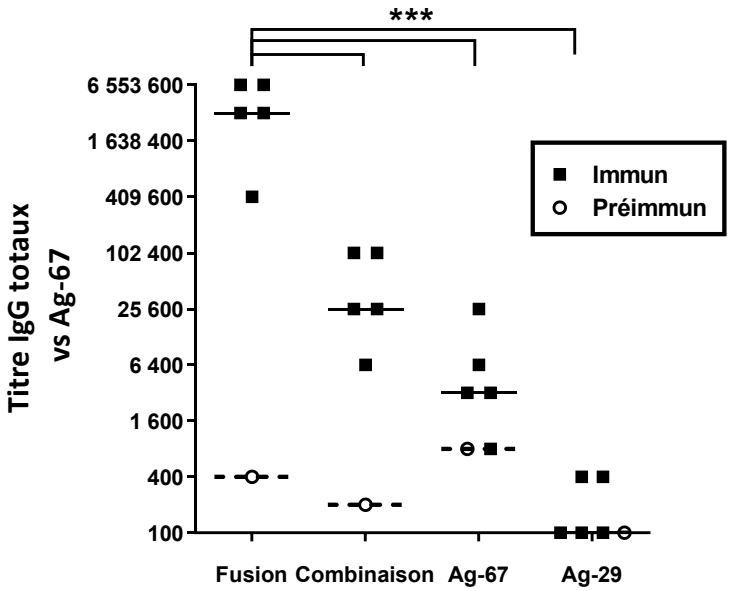
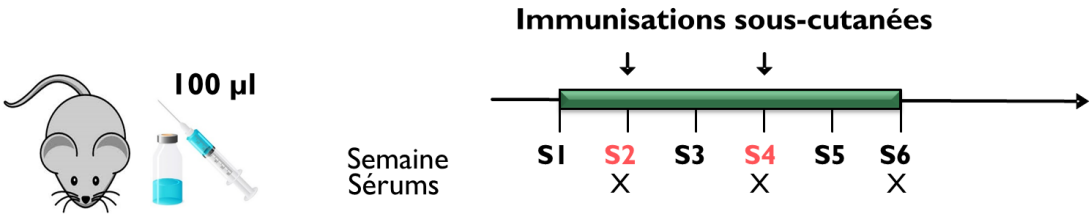


Figure 4.6. Amélioration de la réponse humorale spécifique par la fusion de protéines importantes pour la virulence de *S. aureus*. Titres d'IgG totaux sériques contre l'antigène SACOL1867 (**Ag-67**) de souris immunisées avec la protéine de fusion (SACOL0029-1867; **Fusion**), une combinaison des protéines séparées (SACOL0029+SACOL1867; **Combinaison**), uniquement la protéine SACOL0029 (**Ag-29**) ou SACOL1867 (**Ag-67**), en quantités molaires équivalentes. Les cercles vides (○) représentent les données pour les titres pré-immuns, les carrés noirs représentent les données pour les titres immuns. Pour les titres pré-immuns, des sérums pré-immuns ont été mélangés de manière égale entre les 5 souris de chaque groupe d'immunisation pour obtenir un titre de pool pré-immun, représenté par un cercle ouvert par groupe. Pour les titres immuns, chaque carré représente le titre d'une souris. Les lignes horizontales représentent les médianes (lignes continues : sérums immuns ; lignes pointillées, sérums préimmuns). Les titres des souris vaccinées dans les groupes fusion, combinaison et 1867 sont significativement plus élevés que les titres pré-immuns ($P<0.001$), et les titres des souris ayant reçu uniquement l'antigène monovalent SACOL0029 ne se sont pas avérés significativement différents des titres du pool pré-immun contre SACOL1867. La signification

statistique entre les titres du groupe de fusion par rapport à la combinaison et les deux groupes de vaccins monovalents est montrée (***: P <0.001).

4.4 Objectif spécifique 2 : Démonstration des avantages de la vaccination par la combinaison de la souche atténuée SCV et d'antigènes sous-unitaires sur la réponse humorale chez la souris

Comme il a été démontré dans la section 4.2, la souche vivante atténuée Δ*vraG*Δ*hemB* possède, en plus de sa capacité à générer une réponse humorale forte, une certaine capacité intrinsèque à générer une production d'anticorps plus balancée vers la réponse Th1, du moins lorsque comparée à une formulation vaccinale purement sous-unitaire. Il a donc été envisagé que ses propriétés immunogènes pourraient être employées à améliorer ou orienter la réponse contre les antigènes importants pour la virulence de *S. aureus* (Allard et al., 2013) préalablement utilisés par notre équipe dans un vaccin sous-unitaire (**Annexe IV**). Cette expérience a donc été effectuée dans le cadre de ces études doctorales dans le but de vérifier cette hypothèse (objectif spécifique 2) et aura permis de contribuer aussi à soutenir le brevet WO2018072031 présenté en **Annexe III**. Les propriétés immunogènes de protéines recombinantes de *S. aureus* codées par les gènes SACOL0442, SACOL0720, SACOL0029 et une fusion des gènes SACOL0029 et SACOL1867 ont été évaluées chez la souris cette fois en combinaison avec la souche vivante atténuée Δ*vraG*Δ*hemB*. En bref, les souris ont été immunisées selon le plan expérimental résumé et illustré à la figure 4.7.



3 groupes :

- gr. 1 (N = 5) **mélange de 5 µg des protéines Ag-720, Ag-442, Ag-29 et Fusion 29-67**
- gr. 2 (N = 5) une dose de bactéries atténuées (Δ*vraG*Δ*hemB*; ΔΔ) (10^5 UFC)
- gr 3 (N = 6) combinaison de protéines et de ΔΔ

Figure 4.7. Plan expérimental de la démonstration de l'amélioration de la réponse humorale contre des antigènes de *S. aureus* avec la souche atténuée Δ*vraG*Δ*hemB*.

Les souris femelles CD-1 ont été réparties au hasard en 3 groupes: le groupe 1 (N=5) a reçu une dose de protéines sous-unitaires mélangées (mixture de protéines, Mix); le groupe 2 (N=5) a reçu une dose de 10^5 UFC de bactéries Δ*vraG*Δ*hemB* (ΔΔ); le groupe 3 (N=6) a reçu une combinaison de protéines et de bactéries atténuées (combinaison). Les souris ont été immunisées par deux injections sous-cutanées dans le cou à deux semaines d'intervalle. La préparation des antigènes sous-unitaires a été effectuée comme précédemment décrit dans la section 4.3; les suspensions de bactéries atténuées ont quant à elles été préparées selon la méthode décrite précédemment (Côté-Gravel et al., 2016), évoquée en 4.2. Ces méthodes sont complètement détaillées dans le brevet présenté en **Annexe III**. Les doses de protéines et de souches bactériennes ont été diluées dans du PBS et administrées dans un volume final de 100 µl pour chaque groupe de souris. La détection des IgG totaux et isotypes IgG1 et IgG2a contre les antigènes Fusion 29-67 et Ag-29 utilisés dans le mix de protéines pour l'immunisation a été effectuée par ELISA selon la méthode précédemment décrite dans la section 4.2.

On peut voir comme l'indique la figure 4.8A que les titres en IgG totaux générés contre certains antigènes hautement immunogènes tels que la fusion 29-67 ne sont pas significativement modifiés par la combinaison avec la souche atténuée ΔΔ. En revanche, lorsqu'on utilise des antigènes moins immunogènes, tels que Ag-29, l'ajout de la souche atténuée améliore

significativement la force de la réponse humorale contre celui-ci (figure 4.8B). De plus, de façon similaire à ce qui avait été observé avec l'antigène IsdH dans la section 4.2 de ce chapitre (figure 4.3), la présence de la souche atténuée permet d'augmenter significativement le ratio IgG2a/IgG1 : dans cette expérience-ci, la combinaison avec le mix de protéines a pu améliorer significativement la qualité de la réponse humorale en augmentant ce rapport contre les antigènes fusion 29-67 (4.8C) et Ag-29 (4.8D) comparé au groupe de souris vaccinées par les protéines seules.

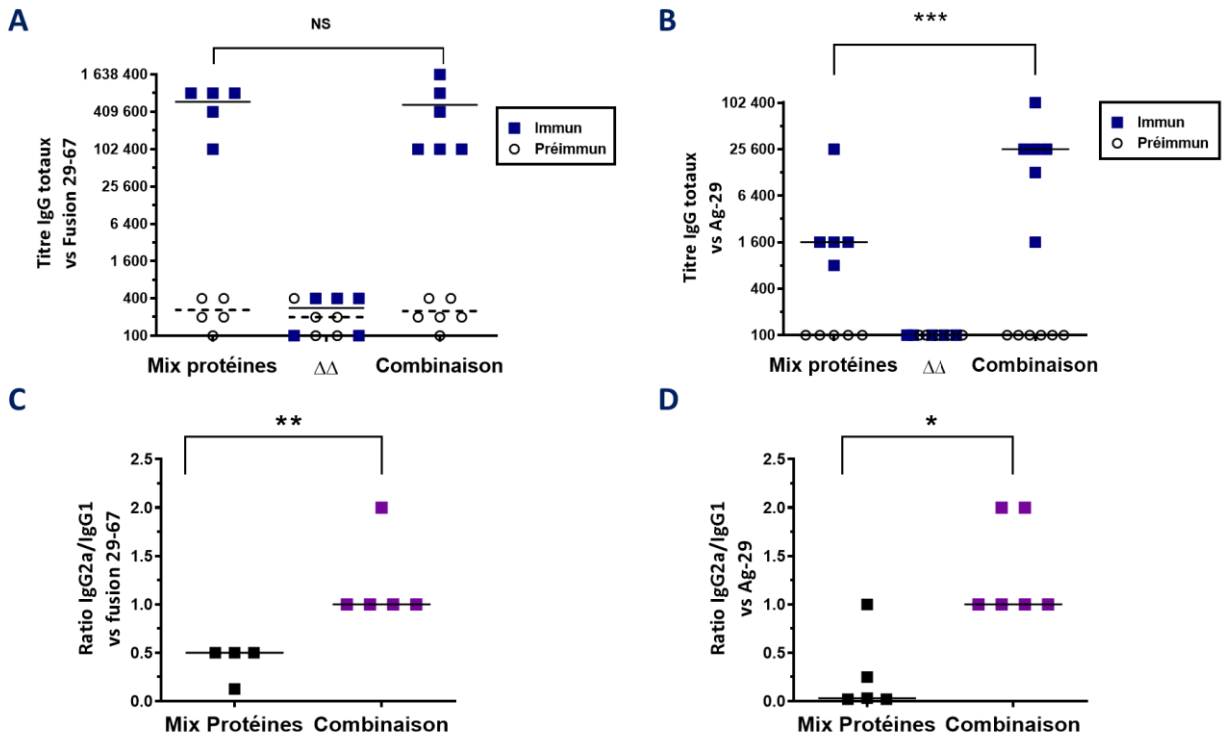


Figure 4.8. Amélioration de la réponse humorale spécifique par la combinaison de la souche atténuée à des antigènes de *S. aureus* sous-unitaires. Titres d'IgG totaux (A-B) ou rapport des isotypes IgG2a/IgG1 (C-D) de souris immunisées avec la mixture de protéines, la souche atténuée (ΔΔ) ou une combinaison des deux. (A) Titres et (C) ratios contre la protéine de fusion 29-67. Les cercles ouverts représentent les données pour les titres pré-immuns, tandis que les carrés de couleur représentent les données pour les titres immuns (A-B) ou les ratios d'isotypes (C-D). Chaque symbole représente le titre ou le ratio d'une souris. Les lignes

horizontales représentent les médianes. La signification statistique entre les groupes comparés est indiquée (*: P <0.05; **: P <0.01; ***: P<0.01; NS: non significatif).

Ainsi, même si une forte réponse en anticorps (fusion 29-67) peut être obtenue par l'immunisation avec différents antigènes sous-unitaires mélangés, l'immunisation par la combinaison de ces antigènes avec une souche vivante atténuée a pu significativement améliorer la force et la qualité de la réponse immune contre *S.* aureus, et ce a) en induisant la production de titres d'anticorps plus élevés contre certains antigènes spécifiques (Ag-29) et b) en orientant la réponse spécifique à ces antigènes vers le type Th1. Ajoutés à la démonstration que la souche atténuée permet d'obtenir une réponse humorale supplémentaire contre d'autres antigènes présents à la surface de la cellule bactérienne (figure 4.2B), ces résultats ont pu démontrer que l'utilisation de la souche atténuée SCV confère de nombreux avantages en vaccination contre le pathogène chronique *S. aureus*.

CHAPITRE 5

DISCUSSION ET CONCLUSION

5.1 Retour sur le contexte et les objectifs de recherche

Ces études de doctorat ont été pour moi l'occasion de poursuivre l'avancement de la caractérisation d'un vaccin vivant expérimental basé sur un SCV hautement atténué amorcée lors de mes études de maîtrise, et tenter de répondre aux nouvelles questions qui avaient été soulevées alors. J'ai donc voulu établir la preuve de concept de l'utilisation de cette souche mutante pour l'immunisation contre le pathogène *S. aureus* en démontrant 1) sa capacité à induire une réponse humorale forte et spécifique; 2) sa capacité à améliorer la réponse contre des antigènes sous-unitaires; 3) les avantages de cette souche vivante comparée à des formulations inactivées. De plus, ce doctorat aura été l'occasion d'explorer de nouvelles pistes pour tenter de comprendre les mécanismes moléculaires à l'origine de la forte atténuation *in vivo* du SCV atténué. J'ai ainsi cherché à 4) comparer les caractéristiques phénotypiques des souches isogéniques parentale et simples mutants (Δ*hemB* et Δ*vraG*) au double mutant SCV atténué (Δ*vraG*Δ*hemB*) et 5) comparer leur transcriptome en présence ou absence de la colistine pour élucider l'importance du système GraXRS-VraFG chez le SCV et son rôle dans l'atténuation.

5.2 La production d'anticorps spécifiques contre *S. aureus* et différents antigènes importants pour sa virulence par le SCV atténué

Les résultats complémentaires abordés dans le **Chapitre 4** ont premièrement constitué cette preuve de concept de l'utilisation du SCV atténué comme moyen de développer une réponse humorale à la fois spécifique, forte et équilibrée contre *S. aureus*, réponse qui pouvait de plus être augmentée proportionnellement à la dose de bactéries utilisées pour immuniser (**objectif**

spécifique 1). Un autre aspect intéressant relevé dans ces premières expériences d'immunisation a été la proportion relativement élevée d'anticorps dirigés contre des antigènes précis du pathogène dans le « pool » complexe d'anticorps anti-*S. aureus* générés. En effet, un écueil possible de l'utilisation de formulations vaccinales telles que des cellules inactivées ou atténuées repose sur le risque de « noyer » la réponse contre des antigènes clés, potentiellement protecteurs, avec des anticorps moins pertinents (Jansen et al., 2013). La comparaison des réponses dirigées contre la protéine IsdH, fortement exprimée *in vivo* par *S. aureus* (Allard et al., 2006; Ster et al., 2010), entre les souris vaccinées avec le SCV vivant *vs* la protéine sous-unitaire a pu démontrer que la génération d'une réponse aux cibles diverses par la souche vivante ne se faisait pas au détriment d'une diminution trop grande des titres d'anticorps susceptibles de protéger, du moins dans le cas de cet antigène. La pertinence d'utiliser la protéine IsdH en particulier pour cette démonstration doit également être mise de l'avant, puisqu'il a été récemment démontré qu'IsdA, une autre protéine du système Isd impliqué dans la séquestration du fer, s'est révélé être hautement exprimée, conservée et immunogène au cours de la mammite bovine (Misra et al., 2018).

Un autre piège important à éviter (abordé dans le **Chapitre 1**) était que les souches de *S. aureus* peuvent être phénotypiquement très différentes; ainsi le risque de développer une réponse incapable de reconnaître les souches cliniquement pertinentes, et donc de protéger sur le terrain, est bien présent. Ce problème potentiel avait été relevé suite aux résultats mitigés obtenus sur le terrain par le vaccin STARTVAC®, utilisant des bactérines de *S. aureus* exprimant le SAAC, une caractéristique qui n'est pas totalement partagée parmi les souches causant la mammite (Prenafeta et al., 2010), bien que les raisons de la faible efficacité de ce vaccin puissent être multiples. Bien entendu, nos essais chez la souris ne peuvent pas pour l'instant fournir d'indication sur le potentiel de protection du SCV atténué. Mais il nous a semblé bon de vérifier en amont de ces essais de protection le potentiel de couverture de la réponse humorale du vaccin atténué à détecter des souches pertinentes. Il a été démontré que l'utilisation du SCV atténué pour immuniser les souris pouvait éliciter une réponse permettant la reconnaissance d'isolats cliniques de mammite génétiquement et phénotypiquement divers. En effet les souches

sélectionnées pour cette démonstration représentaient les principaux types *spa* retrouvés dans les troupeaux laitiers canadiens, des types par ailleurs associés par notre équipe à des capacités de production de biofilm et de persistance d'infection pendant la mammite dramatiquement différentes (Pichette-Jolette et al., 2019). En surcroit, il est bon de mentionner aussi que les expériences comparant le SCV atténué à la formulation tuée associée à la bactérine J5 (**Chapitre 2**), se voulant une représentation de la formulation utilisée par le vaccin STARTVAC®, ont clairement démontré que le vaccin vivant générait des réponses humorales et cellulaires nettement supérieures au vaccin tué, avec ou sans J5. Cette démonstration contrôle réaffirme la pertinence de faire ce genre de caractérisation plus poussée de la réponse avant de faire un *challenge*, ou de passer à des essais chez les grands animaux, dans l'optique de mieux comprendre les facteurs qui influenceront l'efficacité protectrice d'un vaccin. En effet, même après plusieurs années de recherche et de développement, il semble que d'importantes lacunes perdurent dans la compréhension des bases moléculaires de la pathogenèse de *S. aureus* dans l'IIM, l'identification des antigènes staphylococciques induisant une protection et la détermination précise des réponses de l'IMC à l'infection et à la vaccination (Rainard et al., 2018). Combler ces lacunes en orientant la recherche vers des modèles permettant d'élucider plus précisément ces facteurs en amont est une stratégie qui pourrait améliorer en bout de ligne le succès des expériences subséquentes.

5.3 Les avantages d'un vaccin vivant pour augmenter les réponses humorales et à médiation cellulaire

À bien des égards, on peut penser qu'un vaccin bactérien vivant pourrait aussi, en plus d'être une source antigénique riche, agir à titre d'adjuvant de par sa capacité à stimuler l'immunité innée d'une manière étendue et puissante par la présence additionnelle de ligands (tels que des composants génétiques ou de la paroi cellulaire, par exemple) reconnus par les PRRs (Goodridge et al., 2016). L'important niveau de titres en anticorps anti-IsdH obtenus par le SCV atténué, presque comparable à celui obtenu par les souris vaccinées avec IsdH sous-unitaire combiné à l'adjuvant Emulsigen-D (figure 4.2), semblait déjà démontrer un certain pouvoir

d'amplification de la réponse humorale par le SCV atténué. Une autre caractéristique très claire de la réponse générée par le SCV atténué était l'orientation significative de la réponse humorale spécifique vers la voie Th1, démontrée par la proportion plus élevée d'anticorps IgG2a contre les extraits bruts bactériens et la protéine IsdH. Plusieurs raisons pourraient expliquer cette amplification et orientation distincte.

Comme déjà mentionné, les vaccins vivants atténués sont hautement immunogènes parce qu'ils peuvent infiltrer le tissu de l'hôte, continuer à se répliquer et induire une inflammation, similaire à celle de l'infection de par leur capacité à stimuler les PRRs (Cronkite and Strutt, 2018). Cette inflammation est importante car elle contribue à l'attraction de cellules immunitaires innées supplémentaires telles que les neutrophiles, les cellules NK et les monocytes vers le site inflammé. Les cellules inflammatoires recrutées encerclent le site inflammé et libèrent encore plus de cytokines pro-inflammatoires, constituant ainsi une boucle d'amplification et de réorientation de la réponse. Néanmoins, un défi important réside dans la capacité d'imiter cet environnement inflammatoire, sans provoquer un emballement excessif du système immunitaire et les dommages tissulaires associées à une infection (Cronkite and Strutt, 2018). À cet égard, certaines des souris vaccinées par la combinaison de la souche vivante avec la bactérine J5 dans notre étude de comparaison de la souche atténuée aux formulations inactivées (**Chapitre 2**) ont par ailleurs assez mal réagi à l'immunisation, possiblement en raison de l'effet combinatoire trop intense du LPS de la souche de *E. coli* avec le SCV vivant sur l'inflammation, un effet pourtant relevé sur aucune des souris vaccinées seulement par le SCV vivant de tous nos essais. Il est intéressant de rappeler également que lors de l'établissement de l'atténuation du vaccin SCV, il avait été observé que l'IIM expérimentale de souris par le SCV double mutant avait généré un recrutement de neutrophiles élevé, détectable par l'augmentation significative des concentrations de myélopéroxydase (MPO) dans la glande, mais sans causer de rougeur et d'inflammation visuelle importante des glandes comme la souche WT, plus cytotoxique (Côté-Gravel et al., 2016, et **Annexe II**). Il semblerait ainsi que cet effet pro-inflammatoire dicté par l'activation du SI inné par le vaccin vivant puisse jouer un rôle. Il n'est pourtant pas à exclure que d'autres facteurs aient pu entrer en jeu : les micro-organismes vivants fournissent une

stimulation antigénique continue, laissant suffisamment de temps pour monter efficacement la réponse. Dans le même ordre d'idées, il est très possible également que les antigènes présents à la surface ou dans le vaccin vivant aient été moins sensibles à la dégradation que les protéines sous-unitaires.

Une question soulevée par ces résultats était de savoir si cette amplification/orientation de la réponse pourrait être transférée et redirigée contre des antigènes spécifiques de *S. aureus* supplémentaires, et ainsi réellement considérer ces caractéristiques du SCV atténué comme des qualités d'adjuvant. Cette hypothèse pouvait être vérifiée simplement en utilisant le SCV en combinaison avec ces antigènes sous la forme sous-unitaire, et en comparant les réponses obtenues contre ceux-ci entre les souris vaccinées par la combinaison, les protéines seules ou le vaccin vivant seul. Il a été intéressant de constater que l'effet d'amplification de la réponse humorale a pu être établi contre un des antigènes les moins immunogène, soit SACOL0029 (Ag-29), mais pas contre l'antigène fusion 29-67, possiblement en raison de son immunogénicité déjà gonflée par la forme de fusion, mais sans nuire à celle-ci. L'effet de réorientation vers la voie Th1 par le ratio IgG2a/IgG1 était néanmoins clair contre les deux antigènes testés, confirmant du même coup que les qualités du vaccin atténué peuvent en effet être transférées pour améliorer la réponse contre des antigènes sous-unitaires additionnels (**objectif spécifique 2**). Des doses moins élevées du vaccin vivant ont été utilisées pour ces expériences, ce qui a peut-être permis de mieux distinguer l'effet avantageux de la souche vivante dans la combinaison, sans provoquer d'effet de dilution de la réponse contre les antigènes testés, et sans générer de réponse détectable contre ceux-ci dans le groupe vacciné seulement par celle-ci (bien qu'il aurait été moins probable que ces antigènes soient exprimés par le SCV double mutant chez la souris).

Le premier article présenté dans ce document (**Chapitre 2**) a pu poursuivre la démonstration des avantages de l'utilisation de cette souche SCV vivante pour l'amélioration de l'IMC, qui pourrait être cruciale pour combattre un pathogène chronique et hautement adapté à l'évasion de la réponse de l'hôte tel que *S. aureus* (Côté-Gravel et Malouin, 2019). Trouver de nouvelles

pistes pour mieux comprendre comment certaines formulations vaccinales parviennent à éliciter ou non les fonctions effectrices susceptibles de fournir la protection contre les infections à *S. aureus* n'est pas mince affaire. Une autre cible (**objectif spécifique 3**) de ces études doctorales visait entre autres à caractériser et comparer les réponses humorales et à médiation cellulaires élicitées par le vaccin vivant à celles obtenues avec une formulation inactivée employée dans un vaccin commercial contre la mammite. Cet objectif a pu être atteint par la démonstration de l'avantage du vaccin vivant sur le vaccin inactivé, principalement comme on l'a vu par sa capacité à générer des titres nettement plus élevés d'anticorps anti-*S. aureus*, et par la confirmation de sa capacité à stimuler une IMC de type Th1/Th17. Même si les premières caractérisations de la réponse humorale (**Chapitre 4**) avaient déjà relevé une proportion plus élevée d'anticorps de type IgG2a spécifiques chez les souris immunisées avec la souche vivante, celle-ci ne constituait pourtant qu'un indicateur indirect de l'orientation de la réponse vers des fonctions effectrices de l'IMC. Comme abordé dans la section 1.4.4 de ce document, la partie Fc des anticorps IgG2a interagit avec des composants du complément et les récepteurs Fcγ activateurs (Nimmerjahn et Ravetch, 2008) avec une forte affinité. Cette interaction peut permettre l'activation des fonctions effectrices médiées par les récepteurs Fc, qui incluent la stimulation de la cytotoxicité à médiation cellulaire dépendante des anticorps et de l'opsonophagocytose par les macrophages. Il n'en demeure pas moins que cette activation plus forte de l'IMC par le SCV atténué devait être plus directement démontrée. Les résultats de lymphoprolifération des splénocytes de souris (**Chapitre 2**) ont pu dévoiler une meilleure preuve de l'activation de ces effecteurs. En effet, puisque les lymphocytes T de la rate ont effectivement été activés par la réexposition aux antigènes de *S. aureus*, ceux-ci sont susceptibles, par leur profil de sécrétion cytokinique (IFN-γ et IL-17), d'augmenter à la fois la prolifération des acteurs de ces fonctions effectrices et la production par les lymphocytes B d'IgG2a spécifiques à ces antigènes. Un autre aspect révélé par ces résultats a été l'influence de l'ajout de la bactérine J5 sur les réponses des groupes vaccinés par le SCV atténué vivant ou tué à la chaleur. Même si cette différence ne s'est pas révélée significative, on peut relever que le groupe de souris vaccinées par la combinaison SCV inactivé avec J5 a semblé être désavantagé dans la force de sa réponse humorale spécifique à *S. aureus* comparativement au

groupe ayant reçu seulement le SCV inactivé. En plus des conséquences néfastes sur l'inflammation de la combinaison de J5 avec le SCV vivant précédemment abordées, on a pu démontrer que l'ajout de J5 diminuait significativement l'avantage du SCV seul sur le ratio IgG2a/IgG1 ainsi que la production de cytokines IL-17A et IFN-γ des splénocytes proliférés des souris testées. Ces résultats pourraient indiquer une possible réorientation de la réponse vers la voie Th2 par la bactérine J5, ce qui désavantagerait théoriquement les chances de protection contre *S. aureus*.

5.4 Perspectives pour la vaccination contre *S. aureus*

Ces expériences de vaccination chez la souris ont pu faire d'intéressantes démonstrations, qui pourront être très utiles pour poursuivre le développement d'un vaccin efficace contre *S. aureus*, en explorant différents aspects permettant théoriquement d'améliorer les chances de protection contre ce pathogène. Des essais de protection avec le SCV atténué, qui n'ont malheureusement pas pu être lancés dans le cadre de projet, en constituent la suite logique et devront être lancés avant de tirer plus de conclusions sur son potentiel réel d'utilisation pour prévenir les IIMs. L'immunisation sous-cutanée de souris pourrait être suivie d'IIM expérimentales par une souche virulente de *S. aureus*, mais des *challenges* dans d'autres modèles murins d'infections, comme septicémique ou de la cuisse, pourraient aussi être pertinents. En outre, il devrait être envisagé de tester le SCV atténué et caractériser la réponse chez le bovin laitier. Comme il a déjà été relevé au **Chapitre 2**, les expériences *in vivo* sur des modèles murins ne se traduisent pas toujours chez les bovins, tout comme les succès des études précliniques ne se reproduisent pas nécessairement dans les essais cliniques chez l'homme. L'approche privilégiée dans le cadre de ces études a en effet été de mieux comprendre les effets distincts du vaccin SCV atténué dans l'amélioration des réponse spécifiques contre *S. aureus*, que ce soit comparé à des formulations protéiques ou inactivées, avant d'opter pour une façon d'utiliser efficacement ces avantages dans une formulation vaccinale. Des moyens de promouvoir et de mesurer la réponse immunitaire locale dans la glande doivent également être envisagés, puisque la réponse systémique ne se traduit pas toujours par une protection accrue au site d'infection (Dwivedy et

Aich, 2011). Par exemple, il a été démontré que les voies de vaccination peuvent avoir un impact assez élevé sur la réponse immunitaire chez le bovin (Boerhout et al., 2015) et la souris (Gogoi-Tiwari et al., 2015). L'immunisation intramammaire pourrait s'avérer sécuritaire chez la souris, comme démontré par sa colonisation transitoire et temporaire de la glande dans nos études sur l'atténuation de la souche SCV (Côté-Gravel et al., 2016), mais sa sécurité devrait être confirmée chez la vache.

Enfin, sur la base des résultats intéressants obtenus avec le vaccin SCV vivant atténué, qui induisent des réponses immunitaires fortes et équilibrées par rapport aux antigènes protéiques purifiés ou aux bactéries inactivées, le concept de « l'imitation de la viabilité microbienne » représente une nouvelle perspective intéressante. On comprend de mieux en mieux, grâce à des études de plus en plus poussées sur le sujet, les mécanismes moléculaires sous-jacents au succès des organismes vivants à provoquer de meilleures réponses. Il a entre autre été démontré sur des cellules humaines que la reconnaissance de la viabilité bactérienne par le SI inné était entre autres permise par la détection de l'ARN procaryote par le récepteur TLR8 des CPA (Ugolini et al., 2018). Cette reconnaissance s'accompagne de la production de signaux induisant la prolifération de cellules T folliculaires fonctionnelles (T_{FH}) par les CPA, notamment via la sécrétion d'interleukine 12 (IL-12). Les auteurs ont montré que la promotion de la différentiation des cellules T_{FH} était possible à la fois par l'utilisation de bactéries vivantes, d'ARN bactérien nu ou d'agonistes de TLR-8, mais pas via des bactéries tuées ou des agonistes d'autres TLR. Les cellules T_{FH} représentent un sous-ensemble de cellules T CD4 + récemment étudié, maintenant reconnu comme distinct des cellules Th1, Th2 et Th17, et dont la fonction principale est de favoriser la prolifération et la différenciation des cellules B pour la maturation d'affinité des anticorps, la commutation isotypique et le développement d'une immunité humorale de longue durée (Chtanova et al., 2004; Ueno et al., 2015). Chez la souris, une autre équipe a pu également montrer que seule la reconnaissance de bactéries viables ou la complémentation de bactéries tuées par de l'ARN bactérien permettait une cascade d'activation menant à cette différenciation des cellules T_{FH} et leur promotion de la production d'anticorps hautement protecteurs (Barbet et al., 2018).

À cet égard, les vésicules membranaires microbiennes naturelles sont de plus en plus reconnues pour leurs effets immunomodulateurs sur les réponses immunitaires de l'hôte (Ahmadi Badi et al., 2017; Bitto and Kaparakis-Liaskos, 2017; Kaparakis-Liaskos and Ferrero, 2015), et des vésicules à base de membranes artificielles peuvent être utilisées comme systèmes d'administration de vaccins (Bai et al., 2011). On sait depuis plusieurs années que les bactéries Gram-négatives excrètent des vésicules de membrane externe (OMV : *outer membrane vesicles*) suite à leur observation au microscope électronique : la génération de celles-ci se produit par accumulation de phospholipides dans le feuillet extérieur de la membrane externe, suivie de la formation de protrusions de membrane externe qui se « pincent » pour former des vésicules. On sait maintenant que des vésicules similaires sont également libérés par d'autres groupes tels que les archées, les mycobactéries (van der Pol et al., 2015) et les bactéries à Gram positif, dont *S. aureus* (Lee et al., 2009). Une équipe a récemment mis au point un vaccin basé sur des vésicules extracellulaires purifiées d'un mutant de *S. aureus* génétiquement modifié pour exprimer des cytolysines détoxifiées, et a pu montrer que celles-ci étaient immunogènes et protectrices dans un modèle de septicémie mortelle chez la souris (Wang et al., 2018). En outre, ces systèmes, en détenant certains avantages associés aux vaccins atténués (immunogénicité élevée, possibilité d'orienter la réponse), pourraient permettre de contourner les problèmes pouvant être associés aux microorganismes vivants, tels que leur sécurité et donc la plus grande complexité d'approbation pour une utilisation à grande échelle. D'un autre côté, certains défis quant au développement des vaccins à base de vésicules subsistent et doivent aussi être adressés. Par exemple, on remarque parfois la présence d'une réactivité élevée à certains PAMPs tels que le LPS avec les OMVs de bactéries à Gram négatif.

L'utilisation de molécules imitant de mieux en mieux les signatures moléculaires de la viabilité microbienne en vaccination, telles que des agonistes des TLR (Kumar et al., 2019), l'utilisation de vésicules membranaires dérivées des bactéries contenant de l'ARN et/ou des antigènes protecteurs, ou une combinaison de ces éléments, est donc une voie intéressante qui pourrait permettre de contrôler et prévenir efficacement les infections en contournant l'utilisation de bactéries vivantes.

5.5 Importance du système de détection et de résistance aux CAMPs GraXRS-VraFG chez le SCV

Parallèlement aux expériences de vaccination, les résultats obtenus dans notre étude de 2016 (**Annexe II**) quant à l'atténuation du double mutant Δ*vraG*Δ*hemB* dans des modèles cellulaires *in vitro* et d'IIM chez la souris ont soulevé des questions sur le rôle que pouvait jouer le gène *vraG* spécifiquement chez le phénotype SCV qui devaient être adressées dans le cadre de ce doctorat. Notre équipe avait déjà démontré qu'un mutant simple Δ*vraG* était désavantagé dans sa capacité à coloniser la glande mammaire bovine dans des IIM expérimentales (Allard et al., 2013), mais les résultats comparant la capacité d'invasion et de persistance dans les cellules épithéliales de la glande mammaire bovine semblaient montrer une plus grande influence de l'inactivation de *vraG* chez le SCV génétiquement stable Δ*hemB*. La caractérisation phénotypique plus poussée (**objectif spécifique 4**) des quatre souches isogéniques présentée dans l'article 2 de ce document (**Chapitre 3**) a ainsi permis de mieux comprendre les résultats qui avaient été obtenus dans les études précédentes.

Dans les essais de susceptibilité aux antibiotiques, le double mutant SCV Δ*vraG*Δ*hemB* s'est avéré plus sensible à la vancomycine et hautement susceptible à l'indolicidine et à la colistine par rapport au mutant simple *hemB*, tandis que l'effet sur la sensibilité à ces molécules antimicrobiennes cationiques dans la souche Δ*vraG* au phénotype normal par rapport au WT était très modeste, confirmant dans nos conditions le rôle du gène *vraG* dans la résistance aux CAMPs (Falord et al., 2012) mais dans une mesure plus marquée chez le SCV. En cherchant à décortiquer quelles caractéristiques phénotypiques propres aux SCVs pouvaient influencer la susceptibilité à ces molécules, j'ai pu comparer la charge de surface nette, l'hydrophobicité et le potentiel membranaire des souches isogéniques. La susceptibilité moins élevée des SCVs à certains peptides cationiques a souvent été attribuée à la réduction de leur potentiel membranaire (Sendi et Proctor, 2009). Pourtant, un des résultats les plus intéressants de notre modèle de caractérisation phénotypique a été la démonstration d'une corrélation plus directe entre la charge nette de surface cellulaire et la sensibilité aux CAMPs, puisque le double mutant s'est montré

nettement plus susceptibles aux différents CAMPs testés malgré son très faible potentiel membranaire et son hydrophobicité élevée (comparable au mutant *hemB*). À notre connaissance, l'essai de liaison au cytochrome C ou de toute autre mesure phénotypique de la charge nette bactérienne n'avait jamais été effectuée sur des souches SCV de *S. aureus*. Par contre, le lien entre la charge de surface et la résistance intermédiaire à la vancomycine a été démontré chez des souches VISA (Meehl et al., 2007), tout comme le rôle important du système GraXRS-VraFG et son régulon dans cette résistance intermédiaire (Gardete et al., 2012; Hu et al., 2016; Meehl et al., 2007), surexprimé chez ces souches (Howden et al., 2010). Le fait que certaines des caractéristiques phénotypiques typiques (telles que la vitesse de croissance réduite, la pigmentation altérée et l'activité hémolytique réduite) trouvées dans les souches VISA rappellent le phénotype SCV avait été évoqué (Howden et al., 2010), mais un lien entre l'expression des effecteurs du système GraXRS-VraFG et le phénotype SCV n'avait pas à notre connaissance été fait.

Notre évaluation du transcriptome des souches isogéniques (objectif spécifique 5) a pu démontrer que les gènes des effecteurs de la résistance aux CAMPs *vraFG* et *dlt* étaient effectivement surexprimés chez le simple mutant *hemB* en réponse à la colistine comparativement au WT, et fortement régulés à la baisse chez le double mutant comparé à Δ*hemB* avec ou sans colistine, en corrélation avec les caractéristiques phénotypiques de charge et de susceptibilité de ces souches. Puisqu'en absence de colistine, ces gènes étaient plus exprimés chez le SCV simple mutant *hemB* que chez la souche WT, mais dans une moindre mesure (à la limite de franchir le seuil de < Log2FC, donc non-inclus dans nos analyses), le mécanisme précis entrainant l'expression accrue de ceux-ci chez le phénotype SCV en présence de CAMPs est incertain. Ce système de détection semble en tout cas être plus sensible et réactif chez les SCVs, puisque le court traitement à une faible concentration de colistine a davantage affecté l'expression chez les souches SCVs. Une première option pourrait être que les SCVs, contrairement au phénotype régulier (souche WT), ne nécessitent qu'un faible apport supplémentaire en VraG pour augmenter le nombre d'interactions avec GraS et l'activation subséquente de GraR. Ceci entraînerait du même coup une rapide surexpression d'autres

composantes VraFG, en plus des effecteurs Dlt (et MprF), par une boucle de rétroactivation augmentant grandement la différence par rapport au WT. On sait que la détection même des CAMPs et l'activation de GraS est dépendante de la protéine VraG (et de sa boucle extracellulaire) (Falord et al., 2012), donc cette boucle d'activation de sa propre expression aurait une conséquence rapide sur la capacité à détecter et répondre à des concentrations plus faibles de CAMPs, en théorie. Une autre possibilité serait que la distinction provient d'un module GraXRS maintenu dans un état constamment « activé » chez les SCVs en raison d'autres facteurs régulatoires ou phénotypiques présents chez ce phénotype. On sait par exemple que certaines mutations ponctuelles dans GraS peuvent mener à ce genre d'état d'activation accrue, provoquant la résistance intermédiaire à la vancomycine (Howden et al., 2008), mais qu'un tel état puisse être induit par d'autres facteurs chez le SCV serait à démontrer.

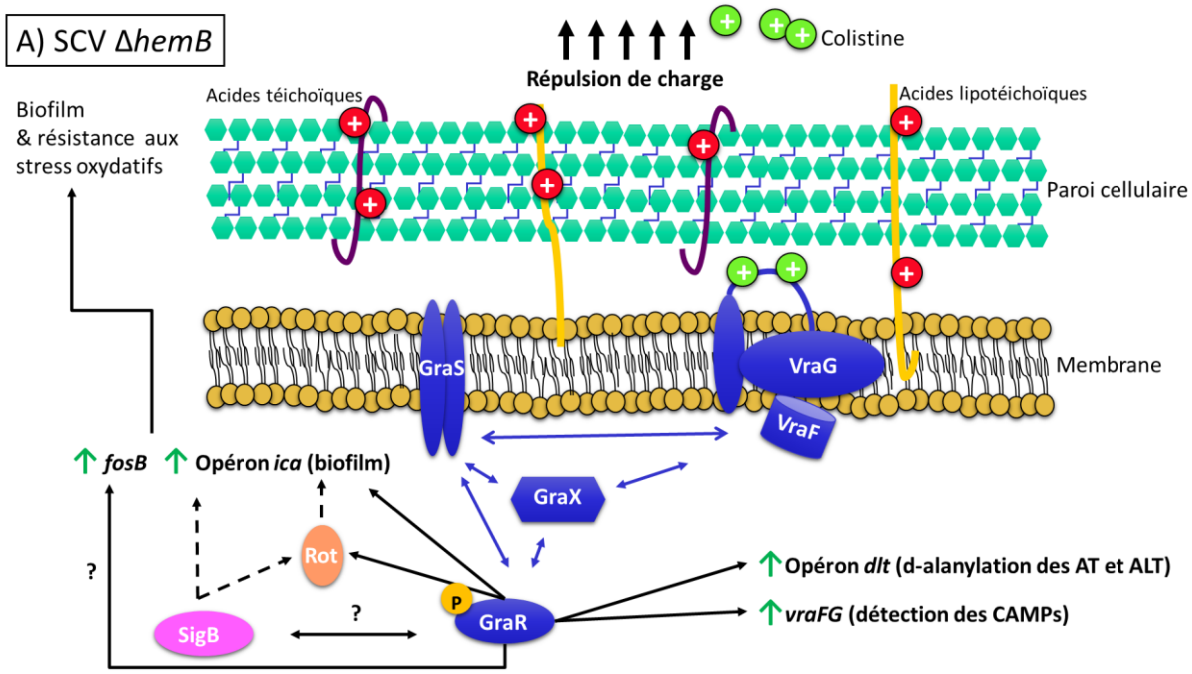
D'autres gènes intéressants sont ressortis différentiellement exprimés dans nos analyses. Il était déjà connu que des mutations dans le système à deux composants VraSR, qui peut détecter et transmettre les signaux de stress de la paroi cellulaire (Chen et al., 2016), sont responsables de l'expression augmentée du régulon de réponse au stress de la paroi cellulaire et de l'épaississement de celle-ci chez les souches VISA (Howden et al., 2010). Tel que déjà constaté chez le SCV (Seggewiß et al., 2006), nos résultats de transcriptomique ont relevé que les deux mutants isogéniques SCVs avaient une expression augmentée de *vraSR* et de nombreux gènes de son régulon, indiquant que la mutation dans *vraG* ne semble pas influencer les gènes de synthèse et de réponse aux stress de la paroi bactérienne. L'épaississement de la paroi de SCVs a déjà été montrée et mesurée (Loss et al., 2019; Onyango et al., 2013), mais il n'est pas clair si cela est présent chez tous les SCVs ou s'il s'agit d'une caractéristique phénotypique stable ou plutôt d'une réponse accrue en réaction aux stress. Il serait donc intéressant de vérifier par microscopie électronique à transmission l'épaisseur de la paroi cellulaire des souches isogéniques, en réponse ou non à certains stress.

Également, il serait important de vérifier l'importance biologique de l'expression diminuée de l'opéron *ica* et du gène *fosB* observée chez le double mutant, à la fois par une contre-vérification

de leur expression par RT-PCR et une élucidation de leur effet respectif sur le phénotype des souches isogéniques. Il avait déjà été observé que GraSR module à la hausse l'opéron *ica*, en plus de contrôler le régulateur global Rot (Herbert et al., 2007), lui-même impliqué de façon importante dans la formation/préservation du biofilm (Mootz et al., 2015). À cet égard, des essais de production de biofilm en présence ou en absence de colistine seraient une bonne avenue pour vérifier si le double mutant est effectivement affecté dans sa production par rapport au simple mutant *hemB*, qui devrait être un plus fort producteur de biofilm que la souche WT (Mitchell et al., 2010). De façon similaire, il serait possible de déterminer la susceptibilité du double mutant à la fosfomycine et au stress oxydatif (par exemple en présence de H_2O_2). En effet, il a été rapporté qu'*in vitro*, FosB utilise sa fonction de Bacillithiol-S-transférase (BST) pour inactiver la fosfomycine, mais ce n'est que plus récemment qu'un rôle possible dans la résistance au stress oxydatif chez l'hôte a été proposé (Posada et al., 2014). Puisque les SCVs se sont avéré être plus résistants au H_2O_2 (Painter et al., 2015), un mécanisme qui pourrait exister chez ce pathogène afin de mieux résister à la flambée oxydative de l'hôte (Painter et al., 2017), il n'est pas impossible que l'expression de *fosB* puisse jouer un rôle dans cette résistance.

Dans un même ordre d'idée, il a été récemment déterminé que le système GraXRS était important pour la croissance de *S. aureus* en milieu acide (Kuiack et al., 2020), et la survie dans le phagolysosome acidifié (Flannagan et al., 2018). Des investigations récentes ont également démontré que ce système de détection joue un rôle supplémentaire dans la virulence de *S. aureus* en interagissant de façon croisée avec un autre TCS; ArlRS. Le système ArlRS est nécessaire pour l'activation transcriptionnelle de l'expression de la protéine A dans les milieu minimum définis (Toledo-Arana et al., 2005; Villanueva et al., 2018). Il a été démontré que le composant senseur GraS est capable de phosphoryler par communication croisée le composant régulateur ArlR du système ArlRS en présence de CAMPs, activant du même coup la production de la protéine A en milieu défini (Villanueva et al., 2018). L'utilisation de colistine pour stimuler GraS a également pu augmenter significativement la production de protéine A d'un mutant Δ*arlS* (Villanueva et al., 2018). On pourrait ainsi penser qu'en étant exposé à la présence de CAMPs ou à l'environnement acide du phagolysosome *in vivo*, GraRS pourrait aussi participer

à moduler l'expression de la protéine A, importante pour la colonisation et l'évasion du SI de l'hôte, mais cela reste à démontrer. Il serait intéressant de déterminer en outre si cette régulation croisée est affectée chez le phénotype SCV, puisque l'expression de la protéine A peut être augmentée chez ce phénotype en raison d'une action réduite du système Agr (Hilmi et al., 2013). Finalement, le schéma illustré à la figure 5.1 brosse un portrait des différences d'expression clés observées dans la présente étude entre le simple mutant *hemB* et le double mutant en présence de colistine, et propose un modèle moléculaire des interactions et régulations à la base du phénotype et de l'atténuation observée chez le double mutant.



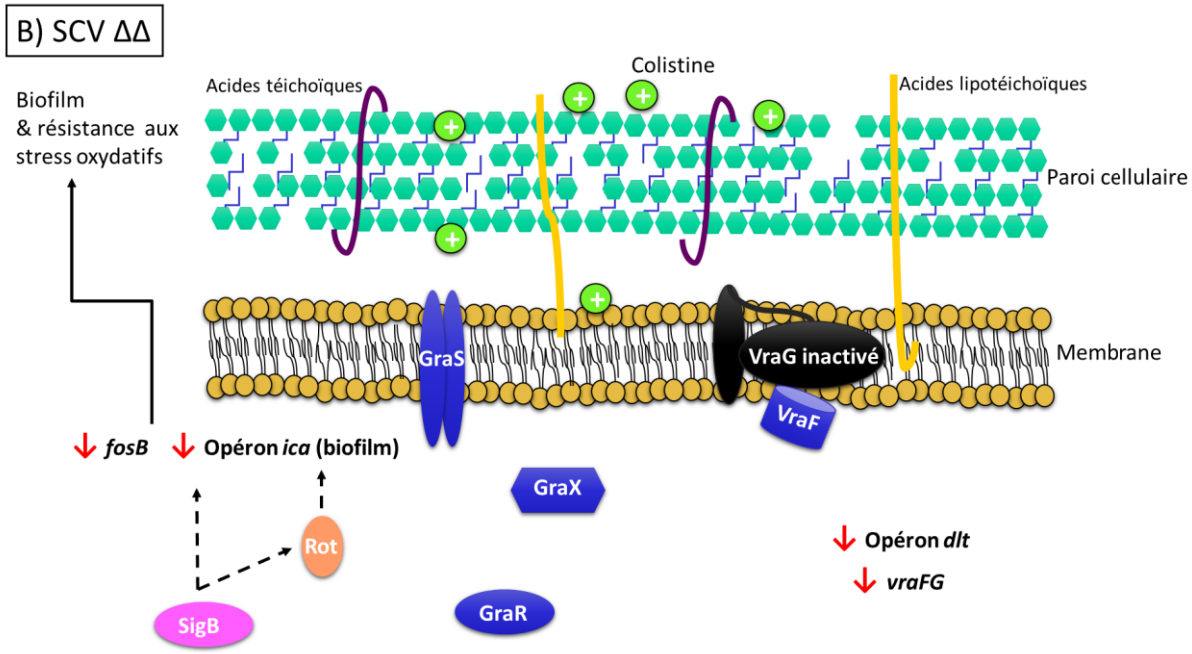


Figure 5.1. Modèle proposé des interactions moléculaires à la base de l'atténuation du double mutant. (A) Chez le simple mutant SCV Δ*hemB*, en présence de colistine (cercle vert +), le système de détection GraXRS-VraFG participe à la détection de celle-ci via la boucle extracellulaire de VraG et son interaction directe avec GraS. La signalisation par la phosphorylation et activation de GraR permet à ce dernier de réguler les gènes et opérons sous son contrôle, tels que *vraFG, dlt*, *ica* et *fosB*, observés comme surexprimés chez ce SCV (↑ vertes). L'expression accrue de *vraFG* pourrait augmenter la sensibilité de détection des CAMPs. L'opéron *dlt* participe à la d-alanylation des acides téichoïques et des acides lipotéichoïques (AT et ALT) de la paroi, augmentant la charge nette positive (résidus positifs, cercles rouges +) de surface et donc la répulsion des CAMPs. GraR augmente également l'expression de l'opéron *ica*, impliqué dans la production de biofilm, qui est également permise via des actions indirectes de SigB (par son action antagoniste du système *agr*) et du régulateur/répresseur de toxines Rot, ce qui pourrait participer aussi à cette répulsion/protection contre l'action des CAMPs. L'expression de *fosB*, impliqué dans la résistance aux stress oxydatifs, est augmentée via des interactions inconnues qui pourraient impliquer GraR, SigB ou d'autres régulateurs globaux de façon directe ou indirecte. (B) Chez le double mutant Δ*vraG*Δ*hemB* (ΔΔ) l'inactivation de VraG compromet la capacité de détecter et signaler la

présence de la colistine, ce qui entraîne une expression significativement diminuée des gènes régulés par GraR (↓ rouges). Puisque VraG permet sa propre expression, cette différence avec le simple mutant Δ*hemB* est d'autant plus exacerbée en présence de CAMPs. La répulsion (charge de surface significativement moins positive) et la production de biofilm, de même que potentiellement la résistance aux stress oxydatifs, sont réduites. Les CAMPs tels que la colistine ou d'autres molécules antimicrobiennes de l'hôte peuvent ainsi exercer leur action sur les membranes ou la paroi cellulaire, et le mutant est ainsi plus susceptible à ces molécules et fortement atténué *in vivo*. Les flèches pleines représentent des interactions directes entre les protéines ou l'activation de la transcription, et les flèches pointillées indiquent des interactions indirectes. Le symbole (?) indique une interaction inconnue ou non-démontrée.

5.6 Conclusion

Ce document s'est voulu une synthèse des travaux de développement d'une nouvelle formulation vaccinale à base d'une souche atténuée *small-colony variant* qui permettrait ultimement d'augmenter la force et les composantes de l'immunité à médiation cellulaire de la réponse contre le pathogène *S. aureus*. La caractérisation des réponses élicitées par ce SCV atténué hautement immunogène permettra de poursuivre le développement d'un vaccin protecteur contre les IIMs à *S. aureus*, que ce soit par son utilisation en combinaison dans une formulation plus complexe ou par la recherche de mécanismes d'imitation microbienne voués à reproduire les avantages de la réponse permise par un tel vaccin vivant.

Puisque l'évasion du système immunitaire de l'hôte et la persistance du pathogène ont démontré être les causes principales du problème que représentent les IIMs sous-cliniques à *S. aureus*, ce nouveau mode de vaccination se devait de prendre en compte les plus récentes connaissances sur les stratégies utilisées par *S. aureus* pour résister aux affronts du système immunitaire ou des traitements. Le maintien de sous-populations SCVs, la survie intracellulaire et « intra-biofilm », et la dynamique de réversion d'un phénotype à l'autre sont des stratégies maintenant

mieux comprises qui pourraient et devront être exploitées pour espérer protéger la glande mammaire contre de nouvelles infections.

En outre, ce projet aura permis ultimement de donner de nouvelles pistes d'explication quant à la grande atténuation du double mutant SCV grâce aux interactions intéressantes mises en évidence entre le phénotype SCV et le système de détection et résistance aux CAMPs GraXRS-VraFG. Cette étude n'a pu dévoiler que la surface du rôle que pouvait jouer ce système chez les SCVs, et il va sans dire que ce sujet de recherche méritera encore beaucoup d'attention dans le futur. Finalement, ces mécanismes moléculaires de réponses au stress utilisés par *S. aureus* lors de son séjour dans l'environnement particulier de la glande mammaire sont importants à élucider d'une part pour justifier l'utilisation d'un vaccin SCV atténué pour immuniser, et d'autre part pour mieux comprendre le rôle clé de ce phénotype dans les infections, afin d'identifier de nouvelles cibles vaccinales et thérapeutiques plus efficaces à contrer ce pathogène si polyvalent et adapté à son milieu.

ANNEXE I

SYMPOSIUM REVIEW: FEATURES OF *STAPHYLOCOCCUS AUREUS* MASTITIS PATHOGENESIS THAT GUIDE VACCINE DEVELOPMENT STRATEGIES

Introduction de l'article et contribution des auteurs

La mammite bovine affecte la santé et le bien-être des animaux ainsi que la production et la qualité du lait, tout en mettant au défi le succès économique des exploitations laitières. *S. aureus* est l'un des agents pathogènes les plus couramment rencontrés dans la mammite clinique, mais il provoque également des infections sous-cliniques, persistantes et difficiles à traiter. En raison des problématiques croissantes associées à l'utilisation d'antibiothérapies, de nombreuses tentatives ont été faites au fil des ans pour développer un vaccin contre cette maladie : pourtant, aucune formulation de vaccin commercialement disponible ne démontre de protection suffisante et un potentiel rentable. De multiples facteurs expliquent le manque de protection, notamment des cibles vaccinales inadéquates, une grande diversité parmi les souches provoquant la mammite, une variation de la réponse immunitaire d'une vache à l'autre et une incapacité à déclencher une réponse immunitaire appropriée pour la protection contre un pathogène très complexe. Le but de cette revue de littérature rédigée pendant mes études doctorales était de résumer les concepts clés liés à la pathogenèse de *S. aureus* et son interaction avec l'hôte dans le contexte particulier de la glande mammaire, ainsi que de brosser un portrait des possibles raisons des échecs en vaccination à ce jour et des stratégies récentes de développement de vaccins pour la prévention et le contrôle des IIMs à *S. aureus*. Pour cette publication, j'ai rédigé la majorité du manuscrit qui a été révisé par mon directeur de recherche, le Pr. François Malouin.

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Symposium review: Features of *Staphylococcus aureus* mastitis pathogenesis that guide vaccine development strategies*

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ABSTRACT

Bovine mastitis affects animal health and welfare and milk production and quality, and it challenges the economic success of dairy farms. *Staphylococcus aureus* is one of the most commonly found pathogens in clinical mastitis but it also causes subclinical, persistent, and difficult-to-treat intramammary infections. Because of the failure of conventional antibiotic treatments and increasing pressure and concern from experts and consumers over the use of antibiotics in the dairy industry, many attempts have been made over the years to develop a vaccine for the prevention and control of *Staph. aureus* intramammary infections. Still, no commercially available vaccine formulation demonstrates sufficient protection and cost-effective potential. Multiple factors account for the lack of protection, including inadequate vaccine targets, high diversity among mastitis-provoking strains, cow-to-cow variation in immune response, and a failure to elicit an immune response that is appropriate for protection against a highly complex pathogen. The purpose of this review is to summarize key concepts related to the pathogenesis of *Staph. aureus*, and its interaction with the host, as well as to describe recent vaccine development strategies for prevention and control of *Staph. aureus* mastitis.
Key words: bovine mastitis, *Staphylococcus aureus*, pathogenesis, vaccine

INTRODUCTION

For decades now, an important amount of significant knowledge and research data have been collected about mastitis in dairy cows. This research has been driven by the increasing need to find effective ways to detect, control, and prevent the disease. Although mastitis can occur following udder trauma or some physiological disorders, it is most often due to bacterial IMI. Despite great progress in our comprehension of host–pathogen interactions, the use of new farm management practices, and the development of novel treatment and prevention tools, bovine mastitis is still the most common disease with the greatest economic impact for the dairy industry (Bar et al., 2008; Ruegg, 2017; Aghamohammadi et al., 2018).

An IMI can manifest in clinical forms of variable threat levels, according to the severity of the symptoms. An IMI can also manifest with a total absence of visible, macroscopic signs of disease, in the form of a subclinical infection. The clinical or nonclinical character of mastitis is mainly influenced by the genus and species of the responsible pathogen (Rainard and Riollet, 2006; Zadoks et al., 2011). Subclinical IMI are responsible for most of the economic problem associated with mastitis (Shim et al., 2004; Petrovski et al., 2006). The vast majority of IMI cases in herds occurs in the subclinical form, and *Staphylococcus aureus* is an important contributor to such subclinical IMI (Reyher et al., 2011).

Treatment and prevention of *Staph. aureus* IMI using antibiotic therapy is difficult. Reported cure rates for *Staph. aureus* mastitis have been shown to vary considerably and seem to depend on many factors at the herd, cow, and bacterial levels, such as herd transmission rates, cow parity, level of SCC, and the genetic background and phenotypic traits of *Staph. aureus* isolates, including their level of biofilm production (Barkema et al., 2006; Ster et al., 2017). Moreover, persistence of the pathogen in the gland can last the lifetime of the animal (Sutra and Poutrel, 1994). Early treatment of new infections can be effective; however, chronically infected cows generally respond poorly to treatment during lactation. In fact, conventional approved treatments rarely achieve a cure rate greater than 20 to 50% and, most of the time, there is a relapse of infections during subsequent lactations, demonstrating that the pathogen is still present or that a new infection can

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arise easily (Dinsmore, 2002; Roy and Keefe, 2012; Ster et al., 2017). In general, extended antibiotherapies or treating dry cows are strategies with a higher probability of cure (Roy and Keefe, 2012; Rainard et al., 2018). There are also risks associated with antibiotherapy, and the public pressure to reduce antibiotic use in animal production is an important incentive to find efficient nonantibiotic control measures for this disease. Of particular concern are the spread of antibiotic resistance genes by horizontal transfer (Li and Zhao, 2018) and the emergence of livestock-associated methicillin-resistant *Staph. aureus* (MRSA) strains that can be transmitted from cows to humans (García-Álvarez et al., 2011; Holmes and Zadoks, 2011).

Thus, preventing infection through vaccination would be a judicious choice for the control of *Staph. aureus* IMI. Ideally, vaccination would eliminate the reservoir of infection in herds, reduce the incidence of new cases of IMI, and improve milk production and quality. Unfortunately, the versatility of the pathogen, which produces a variety of virulence factors, its numerous ways of camouflaging itself from the immune system, and the diversity of strains that can actually cause the disease have prevented successful vaccine development in recent decades (Ismail, 2017; Rainard et al., 2018). However, advances made in the understanding of *Staph. aureus* pathogenesis and on how the host immune system operates during *Staph. aureus* infections open the door to further progress. Figure 1 summarizes host–*Staph. aureus* interactions and provides an overview of the topics of discussion reviewed here, notably the complexity of *Staph. aureus* pathogenesis and some vaccine development strategies.

EFFECT OF *STAPH. AUREUS* IMI

Several families of bacteria can cause mastitis in dairy cows. Overall, staphylococci (*Staph. aureus* and non-*aureus* staphylococci) are the most common mastitis-causing agents, followed by streptococci and *Escherichia coli* (Contreras and Rodriguez, 2011; Reyher et al., 2011; Condas et al., 2017). Pathogens causing IMI can be broadly classified into 2 epidemiological categories: contagious bacteria and environmental bacteria. Coliform bacteria are often described as noncontagious environmental pathogens associated with strong clinical symptoms (Hogan and Smith, 2003). On the other hand, contagious pathogens are mostly transmitted during the milking process and a reservoir of such bacteria can exist within herds. As such, there is real potential that these pathogens cause outbreaks with high incidence rates (Murai et al., 2014). The contagious pathogens include *Staph. aureus*, non-*aureus* staphylococci, *Streptococcus* spp. *Mycoplasma* spp., and *Corynebacterium bovis*. *Staphylococcus aureus* is one of the most important causes of the disease worldwide and the most commonly isolated pathogen in the context of both clinical and subclinical IMI in Canada (Reyher et al., 2011; Naqvi et al., 2018). Milk from infected cows is the main source of transmission of microorganisms from infected cows to their healthy neighbors and this mostly occurs during milking (Olde Riekerink et al., 2008). There is a strong link between transmission of the disease and management quality of the milking facility, which includes teat disinfection procedures and the general cleanliness of the milking equipment used.

Subclinical IMI represent a specific concern because they can stay unnoticed for some time, contribute to the infectious reservoir, and thus increase the risk of transmission. Subclinical IMI can be suspected following an important increase in SCC in milk and confirmed by bacteriology or molecular tests that detect the presence of microorganisms in milk. *Staphylococcus aureus* can alter various milk constituents through production of various hydrolytic enzymes and, consequently, reduce the yield and quality of milk products. The total economic losses associated with *Staph. aureus* clinical and subclinical IMI result from the decrease in milk production and quality, the costs of antibiotic treatments and veterinary fees, and the withdrawal period of cows treated with antibiotics (Halasa et al., 2007; Olde Riekerink et al., 2008; Aghamohammadi et al., 2018).

PATHOGENESIS OF *STAPH. AUREUS* IMI

Several important elements are involved in the virulence of *Staph. aureus* that allow the pathogen to adapt to the mammary gland environment. The pathogenesis of *Staph. aureus* in bovine mastitis has been detailed in several reports over the years (Sutra and Poutrel, 1994; Middleton, 2008; Rainard et al., 2018), so we will summarize here some of the key steps from which vaccine targets can be extrapolated.

Three specific phases are involved in the *Staph. aureus* infection process: (1) adhesion to host cell and extracellular matrix, (2) invasion or penetration into tissues and cells, and (3) evasion of the host immune system (Middleton, 2008). This process is associated with the differential expression of a variety of virulence factors at the different stages of infection. In *Staph. aureus*, the expression of virulence factors is under the control of global regulators such as Agr (accessory gene regulator) and the alternative transcriptional sigma factor SigB (Novick and Geisinger, 2008).

The SigB factor is involved in the response to different stresses and promotes the expression of adhesins and biofilm formation for colonization (Horsburgh et al.,

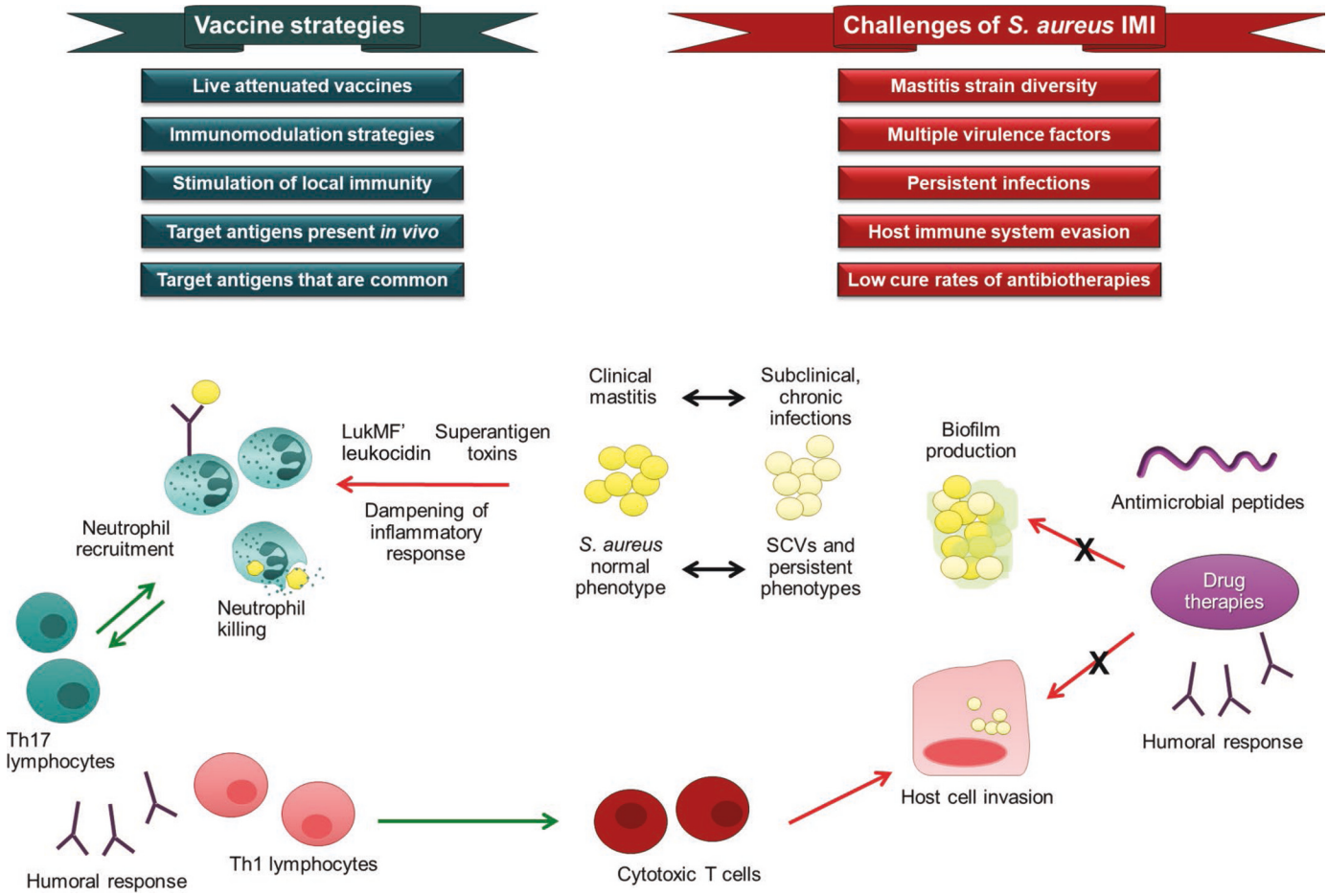

Figure 1. Host–pathogen interactions occurring in *Staphylococcus aureus* bovine IMI and associated challenges for vaccine development. The pathogenesis of *Staph. aureus* is complex, and this bacterium produces several virulence factors that are used for colonization and to escape and modulate the immune system for persistence in the host. One of the challenges in tackling *Staph. aureus* is that the strains that can cause mastitis are diverse and differ in the expression of virulence factors and cell components during IMI, which makes selection of antigens for the development of a vaccine difficult. In addition, through point mutations, highly virulent *Staph. aureus* (bright yellow cells) can adopt a slow-growing phenotype known as "small-colony variants" (SCV; pale yellow cells); SCV are well adapted for long-term persistence in tissues with an improved ability for biofilm formation and internalization into host cells. Biofilm and intracellular living can promote subclinical and chronic infections by protecting *Staph. aureus* from the humoral immune response, antibiotics, and the action of host antimicrobial peptides (crossed red arrows). If such selective pressures for the SCV phenotype are removed, the normal phenotype can be selected back (2-headed black arrow) by its ability to rapidly multiply. Although *Staph. aureus* of the normal phenotype can promote clinical mastitis, superantigen toxins and other mechanisms that are not entirely elucidated grant *Staph. aureus* the capacity to dampen or modulate the inflammatory response. *Staphylococcus aureus* can also counteract opsonization, phagocytosis, and cytotoxic killing by neutrophils through the action of its cytotoxic leukocidin LukMF'. An important challenge for vaccine development will be to elicit an efficient immune response, with mammary-gland localized effectors, and with a strong cell-mediated T-helper (Th)17/Th1 component that will help to neutralize *Staph. aureus* persistence and immune evasion strategies (see text for details on current scientific knowledge). Green arrows denote activation or cooperation, red arrows are antagonistic effects and black arrows represent a change of status.

2002). In contrast, the Agr locus allows transition from the colonization phase to the invasive phase according to a quorum-sensing mechanism (Novick and Geisinger, 2008). When the bacterial cell density increases, Agr decreases the production of surface adhesion proteins; increases the expression of hydrolytic enzymes, exotoxins, and other aggressive virulence factors; and helps detachment from biofilms (Otto, 2013). More specifically, as the bacterial cell density increases, a secreted auto-inducing peptide (AIP), resulting from the coordinated activity of AgrD and AgrB, accumulates in the extracellular milieu and eventually reaches a critical concentration to act on the 2-component system AgrC and AgrA to allow expression of a regulatory RNA (**RNAIII**) that is able to significantly reduce surface protein expression (Novick and Geisinger, 2008). The RNAIII regulator acts directly at the translational level by repressing adhesins as well as *rot* (repressor of tox-

ins) mRNA, and the latter is, in contrast, a repressor of exotoxin synthesis and activator of adhesin synthesis (Boisset et al., 2007). Furthermore, RNAIII permits the translation of *hla* mRNA to allow production of the well-known *Staph. aureus* α-hemolysin, which can lyse several host cell types.

The dynamic regulation of virulence factors orchestrated by the Agr system and other regulators modulates the different phases of *Staph. aureus* IMI. The teat canal represents an important physical barrier that *Staph. aureus* encounters during milking before it is able to spread to the entire mammary gland (Rainard and Riollet, 2006). Following this intrusion, the first step in the colonization of the mammary gland is bacterial adhesion to the host epithelial cells and their extracellular matrix. This attachment allows the bacteria to resist evacuation from the pressure of the milk flux. *Staphylococcus aureus* expresses many virulence factors that are involved in adhesion, including fibronectin (**FnBP**), fibrinogen (**FgBP**) and collagen (CNA) binding proteins, as well as clumping factors (Clf) A and B, and teichoic acids (Brouillette et al., 2003; Mitchell et al., 2008; Middleton et al., 2009). Many such factors are known as MSCRAMM (microbial surface components recognizing adhesive matrix molecules). Biofilm formation is also important in the colonization phase (Otto, 2013). A biofilm is a self-produced extracellular polymeric matrix that can protect the community of bacteria against the action of antibiotics and host immune responses (Hathroubi et al., 2017). After this adhesion phase, *Staph. aureus* synthesizes and secretes factors that allow the invasion, penetration, and destruction of the mammary tissue, including several exotoxins (hemolysins and leukocidins) and various hydrolytic enzymes such as proteases, coagulase, lipases, and hyaluronidases (Middleton, 2008; Suriyaphol et al., 2009). The hemolysins and exoenzymes are implicated in the degradation of the epithelium of the cistern, duct, and alveoli. These microlesions in the mammary gland tissue are responsible for the reduction of milk production, even in the case of subclinical mastitis, when the infection remains unapparent.

Finally, *Staph. aureus* produces a range of factors that allow it to escape but also modulate the host immune system. The efficacy of the host immune system can be impaired by *Staph. aureus* superantigen toxins (Wang et al., 2009), protein A, the polysaccharide capsule, biofilm formation, and the invasion/persistence strategy within host phagocytes as well as nonprofessional phagocytic cells (Middleton, 2008). More particularly, resistance against the action of circulating neutrophils and macrophages, which are the main actors in the immunity in the mammary gland, is believed to be an important factor in the pathogenicity of *Staph. aureus*. Indeed, *Staph. aureus* has the ability to directly counteract opsonization, phagocytosis, and cytotoxic killing. Of particular interest is the leukocidin LukMF', a bicomponent toxin specifically found in clonal lineages of *Staph. aureus* that are associated with bovine mastitis (Vrieling et al., 2015). The LukMF' leukocidin has an AA sequence closely related to that of the human-associated Panton-Valentine leukocidin (PVL; LukS-PV) and LukED and was shown to kill bovine migrating neutrophils through recognition of the CCR1 cell surface receptor with very high specificity and efficacy. It was also established that LukMF' is expressed in vivo during mastitis, although strain-to-strain disparity in expression levels has been reported (Vrieling et al., 2016). In the same study, the severity of mastitis clinical symptoms in experimentally infected cows was correlated with the in vivo expression level of LukMF' in milk. Likewise, a very recent genotypic and phenotypic analysis of clinical isolates from farms in the Netherlands found that *Staph. aureus* strains producing both high and low levels of LukMF' can be found in mastitis samples, of which 22% of the leukocidin-positive *Staph. aureus* isolates displayed a 10-fold higher LukMF' production than the average of the low-producing strains (Hoekstra et al., 2018). These high-producing isolates were cultured significantly more frequently from clinical than subclinical mastitis cases and all belonged to the same *Staphylococcus aureus* protein A (*spa*) type (t543).

DIVERSITY OF *STAPH. AUREUS* STRAINS THAT CAUSE MASTITIS

Staph. aureus Lineages

The immense diversity of strains capable of causing mastitis is a major obstacle in the development of an effective vaccine. Further disparity is present in the phenotypic aspects and the genomic presence of virulence genes among strains capable of causing the disease (Veh et al., 2015). In addition, the level of expression or the subset of *Staph. aureus* genes expressed during infection is profoundly different than that seen in vitro (Allard et al., 2006, 2013) or between strains (Capra et al., 2017). However, despite these discrepancies, the genomic constitution and phenotypic traits of *Staph. aureus* mastitis isolates are the result of a long-term evolutionary adaptation to the bovine host (Bergonier et al., 2014). Notwithstanding, such a host adaptation is very apparent when the different lineages of mastitis-causing strains are compared with human strains (Fitzgerald, 2012; Rainard et al., 2018). Outbreaks of

non-bovine-associated strains have been reported, some of which were caused by strains carrying antibiotic resistance cassettes (Hata, 2016).

Numerous molecular typing methods have been developed for epidemiological studies, including multilocus sequence typing, pulsed-field gel electrophoresis, variable numbers of tandem repeat loci typing (Sabat et al., 2003; Pourcel et al., 2009), and analysis of the polymorphic X region of the protein A gene (*spa*). These typing methods can then be used to associate some strain types with certain genetic and phenotypic characteristics of *Staph. aureus* such as biofilm production, antimicrobial susceptibility, presence of specific virulence genes, and the ability to invade and persist within host cells (Veh et al., 2015; Ster et al., 2017). For instance, *S. aureus* mastitis strains of different sequence types were shown to produce a diversity of superantigen arrays, each having the ability to induce Vβ-specific proliferation of bovine T cells and with some arrays able to stimulate the entire T cell repertoire (Wilson et al., 2018). At the diagnostic level, an interesting approach lies in the potential use of molecular typing to predict the severity and persistence of IMI following identification of the strain causing infection. However, lineage may not always the best marker to predict the severity of infection. Indeed, there could be important differences in the expression levels of toxins and proteases among genetically related *Staph. aureus* strains (Le Maréchal et al., 2011) or between in vitro and in vivo gene expression profiles (Allard et al., 2006). Furthermore, within a single strain, gene expression can change as the infection progresses over time (Jenkins et al., 2015).

Staph. aureus Small-Colony Variants

Over the last decades, a phenotypic variant of *Staph. aureus* has been isolated and characterized in many cases of chronic disease: the small-colony variant (**SCV**). As the name suggests, the main phenotypic feature associated with these subpopulations of isolates is slower growth, leading to the formation of small, nonpigmented and nonhemolytic colonies about one-tenth of the normal size of *Staph. aureus* colonies on solid culture medium (Proctor et al., 2006). These characteristics can be explained by the particular metabolic profile of SCV (Kriegeskorte et al., 2014). The majority of clinical *Staph. aureus* SCV are thought to derive from specific point mutations that lead to deficiencies in the electron transport chain, altering the oxidative phosphorylation process (Kahl et al., 2016).

In addition to the tolerance and resistance of SCV to certain antibiotics, which can be explained by their slow growth and altered metabolism, a specific transcriptional signature allows expression of a set of virulence genes associated with persistence in the host. Small-colony variants have increased capacity for biofilm production (Mitchell et al., 2010; Singh et al., 2010) and an improved ability to invade and survive within host cells compared with prototypic *Staph. aureus* (Kalinka et al., 2014; Löffler et al., 2014). As mentioned above, the expression of virulence factors associated with dissemination and tissue invasion in prototypic *Staph. aureus* is, for the most part, under the control of the quorum-sensing mechanism (the Agr system). On the other hand, because of the slow growth of SCV, SigB preferentially influences the expression of adhesins and biofilm formation in SCV (Mitchell et al., 2013). It is generally accepted that SCV have increased ability to adhere and invade host cells due to high expression of FnBP and, once internalized in host cells, the intracellular persistence of SCV is due, at least in part, to decreased expression of α-hemolysin compared with prototypic *Staph. aureus* (Tuchscherr et al., 2011). Interestingly, although the modified expression of FnBP and α-hemolysin is in fact attributable to the opposing influence of SigB and Agr, Agr could not promote the expression of α-hemolysin in absence of a functional transport chain, as is generally the case for SCV (Pader et al., 2014). Upon selective pressure (e.g., antibiotic treatment, the host immune response, or the intracellular milieu), SCV can be generated and, after release of the selective pressure, the prototypic phenotype can occur from the reversed mutation and be selected by its growth performance and dissemination abilities over the SCV phenotype. It was proposed that such a phenotypic switching is intrinsic to *Staph. aureus* pathogenesis and allows establishment of chronic infections (Tuchscherr et al., 2011).

Small-colony variants are indeed frequently found in chronic infections (Kahl et al., 2016). In recent years, SCV have often been isolated from clinical samples of difficult-to-treat infections such as respiratory tract infections in cystic fibrosis patients, deep post-surgery infections, chronic osteomyelitis, and foreign body infections such as those associated with prosthetic joint and implants as well as from chronic cases of bovine mastitis (Atalla et al., 2008; Alkasir et al., 2013; Yagci et al., 2013; Kalinka et al., 2014; Yang et al., 2018). Certain factors present in these environments may favor the emergence of the persistent phenotype, including antibiotic treatments. Several studies have already reported resistance of SCV to many antibiotics (Vaudaux et al., 2011; Gläser et al., 2014), including aminoglycosides and some cationic peptides that depend on the respiratory chain and membrane potential ($\Delta\Psi$) for their mode

of action (Proctor et al., 2006). Also, the intracellular environment itself may contribute to the emergence of SCV (Tan et al., 2014). Unfortunately, it appears that the prevalence of SCV in the clinical context is still greatly undervalued, in part due to the difficulty in detecting the atypical *Staph. aureus* phenotype in clinical samples (Atalla et al., 2010; Yagci et al., 2013). Recurrent antibiotic treatments and internalization of *Staph. aureus* in epithelial mammary gland cells may indeed represent conditions proper to the generation of the SCV phenotype (Atalla et al., 2010) and potentially explain some of the relatively low cure rates observed for *Staph. aureus* IMI, which often require extended antibiotic therapies (Roy and Keefe, 2012). Furthermore, the host innate immune response for SCV is reduced compared with that observed for the normal phenotype (Ou et al., 2016), and SCV resist the oxidative burst of purified neutrophils and in blood (Painter et al., 2017). Together with the ability of SCV to invade and survive in host cells and their propensity to dynamically revert to an invasive phenotype under more permissive conditions, *Staph. aureus* survival strategies may thus contribute to the establishment of chronic infections by escaping the action of both antimicrobial therapies and host immunity.

VACCINE STRATEGIES

Targeting Bacterial Antigens Expressed During Infection

As mentioned above, *Staph. aureus* strains responsible for bovine mastitis are diverse and can be phenotypically very different. Moreover, *Staph. aureus* gene expression will vary considerably with the phase of infection and in response to the extracellular and intracellular environments. Consequently, a common protective antigen that would be useful against multiple *Staph. aureus* strains has not yet been discovered; very few subunit vaccine compositions have demonstrated substantial benefit to date (Middleton et al., 2009). Also, the selected antigen targets are not necessarily expressed in vivo by all strains or in the mammary gland environment. The in vivo environment can indeed modulate gene expression in *Staph. aureus* (Lowe et al., 1998; Allard et al., 2006). The in vivo expression of some specific known virulence factors was studied previously but it represented a limited selection of targets for vaccine development (Tollersrud et al., 2006). Other studies looked more broadly at *Staph. aureus* proteins and genes that could be expressed in vivo using in vitro culture conditions that could represent the mammary gland or milk environment (Lammers et al., 2000; Taverna et al., 2007).

Because these alternative media and growth conditions do not completely replicate the mammary gland environment or mastitic milk, our research team proceeded to experimentally infect cows to collect milk and bacteria during mastitis, and by a transcriptomic approach, examined genes that were strongly expressed by *Staph. aureus* during infection (Allard et al., 2013). Furthermore, in those experiments, several cows and multiple *Staph. aureus* strains of a variety of *spa* types were used to include host effects as well as strain genotypic and phenotypic diversity. Intramammary infections were followed for 18 d, which allowed us to identify a selection of bacterial genes that were strongly expressed by several *Staph. aureus* strains in a sustained manner during the infection period and in several cows. This approach led to identification of genes and protein candidates for vaccine or drug development.

Among others, gene *vraG* (coding for a putative ABC transporter permease) was shown to be strongly induced by *Staph. aureus* during mastitis (Allard et al., 2013). Because inactivation of that gene by mutagenesis led to a mutant that was significantly attenuated during experimental bovine IMI, the importance of VraG in virulence could be demonstrated (Allard et al., 2013). In fact, VraG seems to be required for cationic antimicrobial peptide sensing and resistance in *Staph. aureus* (Falord et al., 2012). Antimicrobial peptides are an important line of defense for the host against microbial pathogens (Hancock, 2001).

Examining *Staph. aureus* genes that are expressed during bovine mastitis also helped us identify novel drug targets, and we reported that the expression of the essential gene *guaA*, which codes for the enzyme guanosine monophosphate (GMP) synthase and is regulated by a guanine riboswitch (Mulhbacher et al., 2010), could be suppressed by a drug-like small-molecule riboswitch ligand acting as an "antibioswitch." Such a novel class of antibiotic was shown to have some degree of success for the treatment of bovine mastitis (Ster et al., 2013).

In a recent study, an immunoproteomics approach was used to identify antigenic proteins from the surface of *Staph. aureus* to find new potential vaccine candidates (Misra et al., 2018). More specifically, proteins expressed by bacteria grown under iron-restricted conditions to mimic the mammalian host environment were extracted, separated, and blotted followed by specific detection with antibodies from mastitic milk. Thirty-eight *Staph. aureus* proteins were commonly detected by antibodies present in sera of several cows, of which 8 were predicted to be surface-associated and previously determined to be involved in *Staph. aureus* virulence. Of those, 2 surface proteins, the iron-regulated surface determinant protein C (IsdC) and ESAT-6 secretion

system extracellular protein EsxA, were cloned and purified in *E. coli* and then confirmed to be immunoreactive with antibodies from different cows with *Staph. aureus* mastitis. Misra et al. (2018) recognized, however, that this immunoproteomics approach could not distinguish between proteins that are reactive but not protective, although it could represent another effective way to identify new vaccine candidates.

Inactivated and Live Attenuated Vaccines

An inactivated *Staph. aureus* vaccine (StartVac or TopVac; Laboratorios Hipra S.A., Amer, Spain) is commercially available for bovine mastitis. This multivalent vaccine has a *Staph. aureus* component—a bacterin representing strains that express slime-associated antigens part of the biofilm extracellular matrix (Prenafeta et al., 2010). Although the use of a whole bacterin provides a selection of antigens that are suitable for raising an immune response, the success of such an approach is highly dependent on the diversity and type of the *Staph. aureus* strains present in herds. As such, discrepancies in the observed benefit of this vaccine reported in various studies may be attributed to the diversity of *Staph. aureus* strains, which varies geographically, as well as in the degree of slime and biofilm production that may depend on the bacterial growth phase or the environment (Schukken et al., 2014; Bradley et al., 2015; Landin et al., 2015).

Live-attenuated vaccines are still among the most widely used vaccination technologies today. Attenuated vaccines consist of bacterial or viral strains that are deliberately weakened until they are considered harmless or sufficiently less virulent, allowing them to only colonize the target host transiently. Traditionally, live attenuated vaccines have been developed by passage of pathogens under in vitro conditions until they lose their virulence. This empirical approach has been used in the case of the *Mycobacterium bovis* strain BCG for vaccination of humans against tuberculosis. It is now widely recommended that an attenuation strategy be more specific and precise; for example, by using targeted mutations affecting both virulence factors and metabolism (Galen and Curtiss, 2014). Complete gene deletions, compared with mutagenesis of single sites, are considered safer because of the impossibility of reversion, although the acquisition of complete functional virulence genes is theoretically possible via horizontal transfer from environmental bacteria (Frey, 2007).

Live vaccines have several advantages over formulations that use inactivated strains or subunit vaccines that contain purified antigens; for example, (1) they mimic a natural infection and therefore can cause immune responses that are specific, localized, effective, and long lasting (Detmer and Glenting, 2006), and (2) they can prevent the first stages of infection by the pathogen, not just the symptoms of the disease (Frey, 2007). Live-attenuated vaccines also have the significant advantage of offering a broad range of antigenic targets and act intrinsically as what could be called natural adjuvants, by enhancing antigenic presentation through the stimulation of pattern recognition receptors (Griffiths and Khader, 2014). In addition, they can be used as antigenic live vectors for in situ expression of heterologous or modified antigenic proteins via plasmid or chromosomal expression systems. Many intracellular pathogens, such as *Listeria monocytogenes*, have not only been used for their live-attenuated vaccine qualities, but also for their ability to escape the phagolysosome after phagocytosis and to reach the cytoplasm of the infected host cells, where antigen presentation through the major histocompatibility complex (**MHC**)-I for stimulation of cellular immunity is possible (Bruhn et al., 2007). Live-attenuated *Salmonella typhimurium* (i.e., *Salmonella enterica* ssp. *enterica* serovar Typhimurium) strains were also used to deliver recombinant foreign antigens with interesting results (Zheng et al., 2012), notably with the combination of SPI-1 Type 3 secretion system and *Staph. aureus* recombinant EsxA and B proteins. Indeed, this approach allows the recognition of the antigens produced by the bacterium by both MHC-I and MHC-II, because the antigens are delivered by *Salmonella* directly into the cytoplasm.

We recently used a combination of the aforementioned elements to create a novel attenuated mutant of *Staph. aureus* that could be used as a vaccine for bovine mastitis (Côté-Gravel et al., 2016). The strategy took into consideration a critical phenotype part of *Staph. aureus* pathogenesis—SCV formation, which provides the possibility of delivering antigens in the host cell cytoplasmic compartment. Moreover, to avoid reversion to the invasive prototypic phenotype, we used a deletion of the *hemB* gene to create a stable and irreversible SCV phenotype. In addition, as seen in the *Staph. aureus* Small-Colony Variants subsection, although SCV are naturally attenuated in their virulence compared with prototypic strains, their ability to be internalized and to persist within host cells is an important aspect of *Staph. aureus* pathogenesis. Hence, a *vraG* mutation was introduced in the *hemB* SCV background to create an SCV double mutant incapable of sustaining an infection in a mouse mastitis model (Côté-Gravel et al., 2016). Indeed, as described in the Targeting Bacterial Antigens Expressed During Infection subsection, *vraG* is one of the *Staph. aureus* genes that is strongly

expressed during bovine mastitis and that was shown to be an important determinant of virulence in experimental bovine IMI (Allard et al., 2013). As a result, immunization of mice with such a live-attenuated double mutant raised a strong humoral response and antibodies that could also recognize strains from a variety of common mastitis associated *spa* types (Côté-Gravel et al., 2016). Additionally, significantly higher IgG_{2a}:IgG_1 titer ratios, which are indicative of a balanced T-helper (**Th**)1- and Th2-oriented immune response, were detected in sera obtained from mice immunized with the live-attenuated double mutant compared with those of mice vaccinated with the purified IsdH protein.

Immune Responses for Protection

The ability to stimulate a balanced humoral and cellular immune response may be key to achieving protection against *Staph. aureus*. Immunological response to *Staph. aureus* in the mammary gland of ruminants has been reviewed elsewhere (Hughes and Watson, 2018; Rainard et al., 2018). To summarize, the mammary gland of ruminants exploits various types of mechanisms to block the penetration and dissemination of microorganisms. As seen in the Pathogenesis of *Staph. aureus* IMI section, the gland is first protected against the entry of pathogens by structural defenses, among which the physical barrier constituted by the teat canal is undoubtedly one of the most important. The smooth muscle sphincters surrounding the teat canal also prevent leakage of milk by maintaining a tight closure and by producing a keratin layer by the epithelium lining of the duct between milking periods (Aitken et al., 2011). During lactation, the pressure build-up in the udder and the periodic opening of this canal during milking facilitate access of bacteria to the rest of the gland, where they are more likely to proliferate and cause an infection. Once bacteria have entered the cistern, the host innate immunity and adaptive immunity take over and operate in a coordinated manner. This specific response takes action with the presentation of pathogen antigens by specialized antigen-presenting cells (e.g., dendritic cells) and their recognition by naïve circulating B and T lymphocytes. The antigen-specific activated T and B cells then undergo clonal expansion of effector and memory cells through the triggering of specific proliferation cytokines that will influence the type of resulting lymphocyte subsets depending on the type of pathogen, MHC presentation pathway, and influence of cellular regulators during activation (Guzman et al., 2014). These subsets will then correspondingly influence the type of adaptive response that will occur in the gland by regulating specific and nonspecific immune responses (Ezzat Alnakip et al., 2014). During the early stages of infection, the principal actions of innate immunity are unfolded through the pattern recognition receptor pathways and the subsequent outburst of pro-inflammatory cytokines, which include tumor necrosis factor α (**TNF-α**) and IL-1β, among others (Oviedo-Boyso et al., 2007). These specific cytokines promote the rapid recruitment of specialized cellular actors such as the neutrophils and macrophages, which can directly ingest microorganisms by phagocytosis and produce soluble defense components; namely, myeloperoxidase and its generation of reactive oxygen species, antimicrobial peptides, immune-modulating proteins, oligosaccharides, lysozyme, lactoferrin, and complement system components (Aitken et al., 2011; Ezzat Alnakip et al., 2014). Neutrophils are the most important phagocytic cells and are the dominant innate cellular defense against mastitis-causing pathogens in the udder (Rainard and Riollet, 2006), and this seems specifically true against *Staph. aureus* (Spaan et al., 2013). When the innate immunity is not sufficient to clear pathogens, the host adaptive system is activated, and the dominance of neutrophils is shifted to an increasing number of lymphocytes and monocytes (Oviedo-Boyso et al., 2007).

The type of pathogen greatly influences the host response to infection (Petzl et al., 2018). Most coliform species that cause mastitis elicit a marked acute inflammatory response compared with *Staph. aureus* because of the presence of lipopolysaccharide (Ezzat Alnakip et al., 2014). Indeed, the outcome of a coliform-caused clinical mastitis is completely different from one originating from a persistent subclinical *Staph. aureus* IMI (Jensen et al., 2013). In a recent transcriptional analysis of the mammary gland's early response to an experimentally induced bovine mastitis, it was found that *Staph. aureus* IMI, in contrast to those caused by *E. coli*, did not modulate the expression level of any of the nuclear factor (**NF**)-κB factors or their inhibitory IκB regulators (Günther et al., 2017), as previously reported using *Staph. aureus*–stimulated mammary epithelial cells in vitro (Günther et al., 2011). Moreover, activation of the Wnt/β-catenin cascade that provokes suppression of NF-κB signaling and rearrangement of the actin cytoskeleton facilitate invasion of bacteria into host cells. Hence, Günther et al. (2017) proposed that *Staph. aureus* triggers some immune suppression during mastitis. This immunomodulation or dampening of immunity, together with the ability of *Staph. aureus* to invade and persist in host cells, can allow *Staph. aureus* to maintain an effective infection for an extended period. Similarly, another recent study revealed significant transcriptomic differences in microRNA expression of

bovine mammary glands infected with *Staph. aureus* and *E. coli* (Luoreng et al., 2018).

It is suggested that a vaccine that allows the development of a more balanced immunity, with a strong component of cell-mediated immunity, is necessary for protection against pathogens responsible for chronic infections (Kovacs-Nolan et al., 2009). More specifically, it appears that cell-mediated immunity based on Th1 and Th17 type responses may be necessary for protection (Lin et al., 2009; Spellberg and Daum, 2012; Fowler and Proctor, 2014). Thus, new ways to specifically stimulate the Th-1 and Th-17 pathways for the development of effective *Staph. aureus* vaccines are presently being explored (Lacey et al., 2017). It has been shown that antigen-specific Th-1 and Th-17 inflammatory responses are possible following intramammary immunization of cows with a sensitizing protein (Rainard et al., 2013). Another important goal would be to effectively increase the concentration of local or intramammary cell-mediated actors and antibodies in milk. For this purpose, different routes of immunization for vaccine administration, such as intramammary or intranasal inoculations, may provide ways to modulate and accentuate a local response (Boerhout et al., 2015).

ADJUVANTS AND IMMUNOMODULATION STRATEGIES

The development of a subunit vaccine that uses recombinant proteins was stimulated by several advantages, including the cost-effective preparation of specific and relevant antigens, thus eliminating host immune responses toward less important targets or suppressive antigens. Such an approach has, however, prompted a quest for adjuvants that could enhance both humoral and cellular immune responses. The diversity of adjuvants used in different vaccine formulations has been extensively reviewed by several authors (Nicholls et al., 2010; Savelkoul et al., 2015; Burakova et al., 2018). In addition, it has been shown recently that not only the type of adjuvant used for immunization but also the location of a subcutaneous injection can influence the isotypes and titers of antibodies produced in cattle; higher titers were effectively obtained from a prime immunization near the udder with an alum-saponin-oil adjuvant compared with immunization in the neck (Boerhout et al., 2018). The authors of that study hypothesized that with an immunization near the teat, antigens could be taken up by the supramammary lymph nodes, which are more likely to have previously been in natural contact with *Staph. aureus* compared with the prescapular lymph nodes in the neck, and therefore only a single dose of immunization was needed to induce similar effects.

Typical adjuvants include aluminum salts and oil-and-water emulsions of various types that can modulate the release of antigens. Others include pathogen-associated molecular patterns (PAMP) and toll-like receptor agonists that stimulate the innate immune response, as well as some polymers that can either carry antigens or act as immunostimulants on their own. Chitosan is one such a polymer that shows interesting properties. In a recent study, a chitosan-based formulation injected into the teat canal was shown to promote immune cell migration and accelerate mammary gland involution at dry-off, and it was proposed as an alternative to dry cow therapy (Lanctôt et al., 2017). Chitosan is composed of D-glucosamine and *N*-acetyl-D-glucosamine units linked by β-1,4-glycosidic linkages, and its biological activity depends on its molecular weight and the degree of deacetylation. Interestingly, our group demonstrated that a 2.6-kDa chitosan (98% *N*-deacetylated) possessed antibacterial activity against *Staph. aureus* and could help reduce antibiotic use in dairy farms (Asli et al., 2017).

Approaches attempting direct immunomodulation of the udder by cytokines and other response modulators could represent alternatives to vaccination; such studies were reviewed by Petzl et al. (2018). A commercially available example of such an immunomodulator is the granulocyte colony stimulating factor pegbovigrastim (Imrestor; Elanco Animal Health, Greenfield, IN) that is proposed to help restoring neutrophil function during the periparturient period; that is, when cows are most susceptible to mastitis (Canning et al., 2017).

Finally, based on our own experience with live-attenuated strains as vaccines, which induce strong and balanced B and T cell immune responses compared with purified protein antigens, the concept of using microbe-mimicking or microbe-derived membrane vesicles for antigen delivery represents an attractive new perspective. Indeed, naturally occurring microbial membrane vesicles are increasingly recognized for their immunomodulatory effects on host immune responses (Kaparakis-Liaskos and Ferrero, 2015; Ahmadi Badi et al., 2017; Bitto and Kaparakis-Liaskos, 2017), and engineered-membrane vesicles can be used as vaccine delivery systems (Bai et al., 2011).

CONCLUSIONS

Bovine mastitis is the most common reason for antibiotic use in dairy farms. In the One Health concept, which acknowledges the interconnection between human and animal health and the environment, vaccines play a key role in disease prevention and are part of the effort to reduce the use of antibiotics. The challenges in the development of a vaccine for prevention

of *Staph. aureus* mastitis are several. In this review, we underscored the complex pathogenesis associated with this microorganism, which presents invasive and persistent phenotypes. Added to this is a large diversity of mastitis-causing strains, which reduces the probability of finding a universal vaccine. However, selection of relevant antigenic targets for vaccine development has been greatly aided by omics technologies but the need to stimulate both humoral and cellular immunity responses in order to control this bacterium requires innovative adjuvant or immunomodulatory strategies. Overall, a vaccine that offers full protection against *Staph. aureus* IMI is perhaps idealistic at this time but current and future approaches that could reduce disease prevalence should be viewed as important contributions in the field because of their direct effects on antibiotic use in dairy farms.

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ANNEXE II

CHARACTERIZATION OF A *VRAG* MUTANT IN A GENETICALLY STABLE STAPHYLOCOCCUS AUREUS SMALL-COLONY VARIANT AND PRELIMINARY ASSESSMENT FOR USE AS A LIVE-ATTENUATED VACCINE AGAINST INTRAMMAMARY INFECTIONS

Introduction de l'article et contribution des auteurs

Cet article, dont une première version avait été rédigée pendant mes études de maîtrise, a pu être complété et bonifié par de nouveaux résultats d'immunisation effectués pendant la première année de ce doctorat. Comme première étape vers le développement d'un vaccin vivant, cette étude décrit l'élaboration d'un nouveau mutant atténué de *S. aureus* tirant parti du phénotype SCV. Un SCV génétiquement stable a été créé par la suppression du gène *hemB* altérant sa capacité à s'adapter et à revenir au phénotype invasif. Une atténuation supplémentaire a été obtenue par inactivation du gène *vraG* qui avait précédemment été démontré par notre équipe comme important pour la virulence totale pendant les IIM chez les bovins. Ce mutant s'est révélé comme étant grandement atténué dans un modèle d'infection de cellules épithéliales de la glande mammaire dans un modèle *in vivo* d'IIM murin. La vaccination de souris à l'aide d'injections sous-cutanées du double mutant a pu éliciter une forte réponse immunitaire capable de reconnaître différents isolats de *S. aureus* d'intérêt clinique. Ces résultats d'immunisation et d'atténuation auront ultimement mené à la poursuite des deux axes de recherche principaux de mes études doctorales.

La conception du projet de recherche revient au Pr. François Malouin, Céline Ster, Eric Brouillette et moi-même. Les expériences ont été réalisées par moi, Eric Brouillette et Natasa Obradović. J'ai effectué les analyses avec l'aide d'Eric Brouillette et j'ai rédigé le manuscrit, qui a ensuite été révisé par mon directeur le Pr. Malouin et mon co-directeur, Brian Talbot.

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PLOS ONE

RESEARCH ARTICLE

Characterization of a *vraG* Mutant in a Genetically Stable *Staphylococcus aureus* Small-Colony Variant and Preliminary Assessment for Use as a Live-Attenuated Vaccine against Intrammamary Infections

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Abstract

Staphylococcus aureus is a leading cause of bovine intramammary infections (IMIs) that can evolve into difficult-to-treat chronic mastitis. To date, no vaccine formulation has shown high protective efficacy against *S. aureus* IMI, partly because this bacterium can efficiently evade the immune system. For instance, *S. aureus* small colony variants (SCVs) have intracellular abilities and can persist without producing invasive infections. As a first step towards the development of a live vaccine, this study describes the elaboration of a novel attenuated mutant of *S. aureus* taking advantage of the SCV phenotype. A genetically stable SCV was created through the deletion of the *hemB* gene, impairing its ability to adapt and revert to the invasive phenotype. Further attenuation was obtained through inactivation of gene *vraG* (SACOL0720) which we previously showed to be important for full virulence during bovine IMIs. After infection of bovine mammary epithelial cells (MAC-T), the double mutant (Δ*vraG*Δ*hemB*) was less internalized and caused less cell destruction than that seen with Δ*hemB* and Δ*vraG*, respectively. In a murine IMI model, the Δ*vraG*Δ*hemB* mutant was strongly attenuated, with a reduction of viable counts of up to 5-log10 CFU/g of mammary gland when compared to the parental strain. A complete clearance of Δ*vraG*Δ*hemB* from glands was observed whereas mortality rapidly (48h) occurred with the wild-type strain. Immunization of mice using subcutaneous injections of live Δ*vraG*Δ*hemB* raised a strong immune response as judged by the high total IgG titers measured against bacterial cell extracts and by the high IgG2a/IgG1 ratio observed against the IsdH protein. Also, Δ*vraG*Δ*hemB* had sufficient common features with bovine mastitis strains so that the antibody response also strongly recognized strains from a variety of mastitis associated *spa* types. This double mutant could serve as a live-attenuated component in vaccines to improve cell-mediated immune responses against *S. aureus* IMIs.

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Introduction

Staphylococcus aureus is a major human and animal pathogen that can cause high morbidity, acute infections, as well as difficult-to-treat chronic forms of diseases. Among factors that can explain the failure of antibiotherapy and the tendency to cause chronic infections, many have noted the pathogen's multifaceted virulence, predominantly its abilities to impair or elude host immune responses by toxin secretion [1,2], formation of biofilm [3] and survival in non-phagocytic host cells, which may shield the pathogen from the action of host immune system and antibiotics [4]. Furthermore, incidences of *S. aureus* infections are becoming more worrisome with the emergence of multiple antibiotic resistant strains [5,6]. Consequently, there is an urgent need to find potent new strategies to control this pathogen.

As for today, bovine mastitis is still an important problem for the dairy industry, and *S. aureus* is the most frequent pathogen in all combined cases of clinical and subclinical intramammary infections (IMIs) [7]. Subclinical IMIs in particular can be a real concern: they often stay unnoticed by producers, are highly transmissible during milking and thus result in chronic infections that can persist for the life of the animal [8]. Over time, they can generate tissue damage that rapidly leads to a decrease in milk production and quality [9].

The development of vaccines for the prevention and control of *S. aureus* IMIs has been extensively investigated, although no formulation has demonstrated high protective efficacy to date. According to several reviews of the different commercially available and experimental vaccine formulations, this lack of protection is possibly caused by inadequate vaccine targets [10,11], high diversity among strains capable of provoking mastitis [10,12,13] or the failure to elicit an appropriate immune response [14–16]. It is increasingly understood that immunity solely based on vaccine-induced antibodies may be important, but is however insufficient for inducing protection against *S. aureus* [10,11]. It appears that cell mediated immunity (CMI) based on Th1 and Th17 type responses may be necessary to complete the protection [15–18].

In a previous study, we used a DNA microarray approach to uncover *S. aureus* genes that were highly expressed during bovine IMIs [19]. One gene (*guaA*) was shown to be a good target for a new drug therapy [20], and other genes were further investigated as vaccine candidates. Gene *vraG* (SACOL0720) was shown to be likely induced by the growth of *S. aureus* in fresh milk both *in vitro* and *in vivo*. The importance of gene *vraG* in *S. aureus* virulence was also demonstrated by the significant attenuation of growth observed for the gene inactivation mutant during bovine IMI [19].

It is now recognized that *S. aureus* small colony variants (SCVs) add important contributions to chronic infections and therapy failures. This may be attributed to the particular features of SCVs that make this phenotype adapted for long-term persistence in host tissues via expression of a distinct set of virulence factors [21], and that also allow survival in host cells [22,23]. Since SCVs have an improved ability for internalization into cells [4,24,25] and can colonize the host without generating invasive infections or tissue destruction [26,27], we hypothesized that these features could be of value in the development of genetically attenuated *S. aureus* strains. The use of *S. aureus* live-attenuated bacteria as vaccines represents an interesting approach to improve immune responses. Live-attenuated organisms that mimic natural infections stimulate the immune system in a powerful manner, eliciting broad and robust immune responses that increase serum and mucosal antibodies as well as effector and memory T cells which act synergistically to protect against disease [28,29].

In this study, we generated a *vraG* mutation in a SCV background to create an attenuated strain for vaccine purposes. Inactivation of gene *vraG*, should prevent cationic peptide resistance [30–32] and reduce virulence [19], while inactivation of gene *hemB* creates a stable SCV and prevents reversion to the invasive phenotype, a phenomenon normally seen during *S.*

aureus infections [33]. We evaluated the persistence of the double mutant in a bovine mammary epithelial cells and demonstrated its attenuation and safety in a murine IMI model. We also report some immunogenic properties of this vaccine strain. This work is a first step in the proof of concept needed for the development of a live-attenuated vaccine for immunization and protection against *S. aureus* IMIs.

Materials and Methods

Ethics statement

The animal experiments were conducted following the guidelines of the Canadian Council on Animal Care and the institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke. The institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke specifically approved this study.

Bacterial strains and growth conditions

Strains used in this study are listed in Table 1. *S. aureus* ATCC 29213 and its isogenic mutant Δ720 were previously described [19]. Strain Δ720 is an intron insertion mutant of gene *vraG* that was renamed in this study Δ*vraG* for clarity. For the immunological tests, we selected four different bovine mastitis isolates corresponding to some of the predominant *S. aureus spa* types found in Canadian dairy herds and elsewhere in the world [13,34]. Strain SHY97-3906 (*spa* t529) was isolated from a case of clinical bovine mastitis that occurred during the lactation period, and CLJ08-3 (*spa* t359) was originally isolated from a cow with persistent mastitis at dry-off [19]. Strains Sa3151 (*spa* t13401) and Sa3181 (*spa* t267) were obtained from the Canadian Bovine Mastitis and Milk Quality Research Network (CBMMQRN) Mastitis Pathogen Culture Collection, and were isolated from cases of subclinical IMIs. Unless otherwise stated, *S. aureus* strains were grown in tryptic soy broth (TSB) and agar (TSA) (BD, Mississauga, ON,

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant details	Source or Reference
Strains		
S. aureus		
RN4220	Derivative of 8325–4, Restriction-deficient strain that accepts DNA from *E. coli*	[35]
ATCC 29213	Wild-type, VraG positive, normal phenotype	ATCC 29213
Δ*vraG*	*vraG* (SACOL0720) intron insertion mutant, isogenic to ATCC29213	[19]
Δ*hemB*	*hemB*::Em^r; isogenic mutant of ATCC29213, SCV phenotype	This study
Δ*vraG*Δ*hemB*	*hemB*::Em^r; isogenic mutant of Δ*vraG* SCV phenotype	This study
SHY97-3906	Isolate from a dairy cow with a case of clinical mastitis occurring during the lactation period; *spa* type t529	[19]
CLJ08-3	Isolate from a dairy cow with a case of subclinical IMI persisting through the dry-off period; *spa* type t359	[19]
Sa3151	Isolate from a dairy cow subclinical IMI occurring during the lactation period; *spa* type t13401	This study
Sa3181	Isolate from a dairy cow subclinical IMI occurring during the lactation period; *spa* type t267	This study
E. coli		
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen Life Technologies
Plasmids		
pBT2	Shuttle vector, temperature-sensitive; Ap^{r}Cm^r	[36]
pBT-E	pBT2 derivative, inserted *ermA* cassette; Ap^{r}Cm^{r}Em^r	This study
pBT-E*hemB*	pBT2 and pBT-E derivative, for *hemB* deletion: insertion of ~1000 bp of *hemB* flanking regions on both sides of *ErmA*; Ap^{r}Cm^{r}Em^r	This study

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Canada), and *Escherichia coli* DH5α was grown in LB and LBA medium (BD). The ability of *S. aureus* strains to produce biofilm *in vitro* was evaluated as described before [13]. Whenever required, ampicillin (100μg/ml) (Sigma-Aldrich, Oakville, ON, Canada), chloramphenicol (20 μg/ml) (ICN Biomedicals, Irvine, CA), and erythromycin (10 μg/ml) (Sigma) were added to culture media.

DNA manipulations

Recommendations from the manufacturers of kits were followed for genomic DNA isolation (Sigma), plasmid DNA isolation (Qiagen, ON, Canada), extraction of DNA fragments from agarose gels (Qiagen) and purification of PCR products and of digested DNA fragments (Qiagen). An additional treatment of 1h with lysostaphin (Sigma) at 200 μg/ml was used to achieve efficient lysis of *S. aureus* cells in genomic and plasmid DNA isolations. Primers were designed to add restriction sites upstream and downstream of the amplified products. PCRs were performed using the Taq DNA Polymerase (NEB, Pickering, ON, Canada) for routine PCR or the Q5 high fidelity DNA Polymerase (NEB) for cloning, and cycling times and temperatures were optimized for each primer pair. Plasmid constructs were generated using *E. coli* DH5α (Invitrogen, Burlington, ON, Canada), restriction enzymes (NEB), and the T4 DNA ligase (NEB). Plasmid constructs were validated by restriction digestion patterns and DNA sequencing before electroporation in *S. aureus* RN4220 [35] and in final host strains. Plasmids used in this study are listed in Table 1.

Generation of pBT-E:*hemB* and insertional deletion of *hemB*

Isogenic *hemB* mutants of the ATCC 29213 and Δ*vraG* strains were constructed, in which the *hemB* gene was deleted and replaced by the insertion of an *ermA* cassette by homologous recombination. The temperature-sensitive [36] pBT2-*hemB*:*ermA* (pBT-E:*hemB*) was used in a strategy previously described [37], with some modifications. Briefly, the pBT-E plasmid was constructed by the insertion of an *ermA* cassette between the *Xba*I and *Sal*I sites of the temperature-sensitive shuttle vector pBT2. The flanking regions of gene *hemB* [38] DNA fragments were amplified from *S. aureus* ATCC 29213 and were cloned on both sides of the *ermA* cassette into the plasmid pBT-E. The plasmid was then transferred for propagation into *S. aureus* RN4220 (res-). After bacterial lysis with lysostaphin (200 μg/ml for 1 h at room temperature), plasmid DNA was isolated and used to transform ATCC 29213 and Δ720 by electroporation. For plasmid integration and mutant generation, bacteria were first grown overnight at 30˚C with 10 μg/ml of erythromycin and a 1 μg/ml hemin supplementation (Sigma). Bacteria were then diluted 1:1000 and grown overnight at 42˚C with 2.5 μg/ml of erythromycin and 1 μg/ml hemin. This step was repeated twice. Finally, bacteria were diluted 1:1000 and grown overnight at 42˚C without antibiotics. Mutants with the inactivated *hemB* gene were selected as resistant to erythromycin and sensitive to chloramphenicol, together with a SCV phenotype that can be complemented *(i.e.*, reversion to the normal growth phenotype) by a 5 μg/ml hemin supplementation on agar plates. The deletion of *hemB* in the ATCC 29213 and Δ*vraG* strains was confirmed by PCR and DNA sequencing of the PCR product.

Hemin supplementation in broth culture

To evaluate the capacity of hemin to restore optimal growth kinetics of *S. aureus* Δ*hemB* and the double mutant Δ*vraG*Δ*hemB*, overnight bacterial cultures were diluted to an $A_{600\ nm}$ of approximately 0.1 in culture tubes containing fresh BHI supplemented with hemin (Sigma) added at various concentrations. The A_{600nm} of cultures was monitored at different points in time during the incubation period at 35˚C (225 rpm).

S. aureus infection of bovine mammary epithelial cells

An established bovine mammary epithelial cell (BMEC) line, MAC-T, was used as a cell culture model of infection [39], and was used for the characterization of intracellular infectivity and persistence of *S. aureus* ATCC 29213 and its isogenic mutants. The MAC-T cells were routinely cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), supplemented with 5μg/ml insulin (Roche Diagnostics Inc., Laval, QC, Canada) and 1μg/ml hydrocortisone (Sigma), and incubated at 37˚C in a humidified incubator with 5% CO_2. Cell culture reagents were purchased from Wisent (St-Bruno, QC, Canada).

Forty-eight hours before infection, $1x10^5$ MAC-T cells per ml were seeded on treated CellBIND® 24-well plates (Corning) to obtain 30% confluence. Monolayers were grown to confluence under 5% CO_2 at 37˚C. Six hours prior to infection, monolayers were washed with DMEM and incubated with an invasion medium (IM) (growth medium without antibiotics containing 1% heat-inactivated FBS). Overnight bacterial cultures were diluted 1:20 in fresh TSB and grown to mid-logarithmic growth phase, then washed with PBS and diluted in IM to a multiplicity of infection of 10. Invasion was achieved by incubating monolayers with bacteria for 3 h. Monolayers were then washed with DMEM and incubated with IM containing 20 μg/ml lysostaphin to kill extracellular bacteria. The use of lysostaphin to kill extracellular normal and SCV *S. aureus* was previously validated in cell invasion assays [24,39]. The treatment was allowed for 30 min before the determination of intracellular CFUs after 3h of infection, or the treatment was extended for an additional 12 or 24 h for those later time points. For CFU determination, following extensive washing with Dulbecco's Phosphate-Buffered Saline (DPBS), monolayers were detached with trypsinization and lysed with 0.05% Triton X-100 before PBS was added to obtain a final 1X concentration. The lysate was serially diluted and plated on TSA for CFUs determination.

BMECs viability and metabolic activity assay

To determine the cytotoxic damage inflicted by *S. aureus* ATCC 29213 and its isogenic mutants on MAC-T cells, a cell metabolic activity assay that measures the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) into an insoluble formazan product in viable cells, was performed. The assay followed the method of Kubica *et al.* [40] with some modifications. Briefly, *S. aureus* infection of cells was achieved as described in the persistence assay, but instead of inducing cell lysis after 12 h or 24 h, cells were incubated with 100 μl of the MTT reagent (5 mg/ml) (Sigma) in DPBS for 2 h at 37˚C. Following this, an acidic solvent solution of 16% SDS and 40% PMF, pH 4.7, was added to lyse the cells and solubilize the crystals of formazan overnight. The samples were read using an Epoch microplate reader (Biotek Instruments Inc.) at a wavelength of 570 nm. All assays were performed in triplicate, and control wells with uninfected cells (high viability control) or lysed bacteria-infected cells (bacteria-background control; treated with 0.05% Triton X-100 for 10 min before MTT addition) were included to each plate. The level of metabolic activity was calculated using the following formula:

$$\left(\left(\text{Absorbance of the sample} - \text{Absorbance of bacteria} - \text{background control}\right) / \text{High viability control}\right) \times 100$$

Virulence in the mouse mastitis model

The mouse mastitis model of infection was based on previously described work [4,41]. Briefly, one hour following removal of 12–14 day-old offspring, lactating CD-1 mice (Charles River

Laboratories) were anesthetized with ketamine and xylazine at 87 and 13 mg/kg of body weight, respectively, and mammary glands were inoculated under a binocular. Mammary ducts were exposed by a small cut at the near ends of teats and a 100 μl-bacterial suspension containing ~10^2 CFUs in endotoxin-free phosphate-buffered saline (PBS, Sigma) was injected through the teat canal using a 32-gauge blunt needle. Two glands (fourth on the right [R4] and fourth on the left [L4] from head to tail) were inoculated for each animal. Mammary glands were aseptically harvested at the indicated times, weighed and visually evaluated for inflammation. Bacterial burden was evaluated after mechanical tissue homogenization in PBS, serial dilutions, and plating on agar for CFU determination. In additional experiments, homogenized glands were preserved for protein extraction and myeloperoxidase (MPO) activity assays.

Mammary gland protein extraction

Total protein extraction from mammary glands was performed by an optimized method previously described [42], with some modifications. Mammary tissues were homogenized in a buffer containing a final concentration of potassium phosphate of 50 mM, pH 6.0, and hexadecyltrimethylammonium bromide (CTAB) 50 mM (Sigma). The samples were then sonicated, freeze-thawed in liquid nitrogen, and centrifuged at 2000 g for 15 min at 4˚C. Finally, the fat layer was removed by aspiration, and supernatants were saved for a final centrifugation of 15 min at 15 000 g, to discard all cellular debris. Supernatants were distributed in aliquots and kept at -80˚C until used for the enzymatic assays or protein concentration determination as measured by the bicinchoninic acid method (BCA) Protein Assay Kit (Thermo-Scientific).

MPO activity assay

Neutrophil recruitment in mammary tissues was measured by quantification of the MPO enzyme activity by the *o*-dianisidine-H_2O_2 method, modified for a microplate format [43]. In a 96-well microplate, 10 μl of tissue extraction supernatants were incubated with a solution of *o*-dianisidine hydrochloride (167 μg/ml) (Sigma) and 0.0005% H_2O_2 (Sigma) in 50 mM CTAB phosphate buffer 50 mM, pH 6.0. The MPO activity was measured kinetically with intervals of 15 s over a period of 5 min in an Epoch microplate reader at 460 nm. A Unit of MPO was considered as the amount of enzyme that degrades 1 μmol of H_2O_2/min at 25˚C, assuming an absorption coefficient of 11.3 mM^{-1} cm^{-1} at 460 nm for *o*-dianisidine [44]. Results were expressed as units of MPO per g of gland.

Mouse immunizations

The immunogenic properties of the attenuated strain Δ*vraG*Δ*hemB* administered as a live vaccine were evaluated in mice. In preliminary studies, the mice well tolerated intramuscular and subcutaneous (SC) injections of the attenuated strain. The doses of 10^6, 10^7 and 10^8 CFUs and the SC route were selected for subsequent experiments. For the preparation of bacterial inoculum, *S. aureus* Δ*vraG*Δ*hemB* colonies previously grown on BHIA plates were washed twice in ice cold PBS and suspended in PBS containing 15% glycerol, then aliquoted and kept at -80˚C until subsequent use. The viable bacterial counts in the inoculum preparation was validated by serial dilution plating on BHIA. CD-1 mice were randomly divided into 3 groups: group 1 (n = 3) received a dose of 10^6 CFUs; group 2 (n = 3), 10^7 CFUs, and group 3 (n = 3), 10^8 CFUs. Mice were immunized by two subcutaneous injections of bacteria in PBS (100 μl), in the neck, two weeks apart. This live-attenuated formulation was also compared to a subunit vaccine using only the purified staphylococcal IsdH protein as the antigen. The recombinant *S. aureus* IsdH protein was produced in *E. coli* as previously described [45]; mice (n = 6) were immunized by two subcutaneous injections in the neck, three weeks apart, using 20 μg of IsdH

combined to EMULSIGEN®-D (25% v/v) (MVP Laboratories, Inc., Omaha, NE) in a volume of 100 μl. Blood samples were taken just before the priming injection (preimmune serums) and 10–21 days after the boost immunization (immune serums). Blood aliquots were allowed to clot at room temperature for an hour and then centrifuged at 10,000 g for 10 min at 4˚C. The serums were collected and kept at -20˚C until subsequent analysis.

Preparation of *S. aureus* cell extracts

Preparation of *S. aureus* whole cell extracts was done as previously described with some modifications [46]. Briefly, overnight bacterial cultures were diluted 1/1000 in fresh BHI broth, and then incubated at 35˚C (225 rpm) until an absorbance value (OD_{600nm}) of ~ 0.8 was reached. Bacterial cells were centrifuged and pellets were washed in ice-cold PBS twice and suspended with the addition of 5 ml of PBS per ml of pellet. Bacterial suspensions were first treated with lysostaphin (Sigma) (100 μg/ml of pellet) for 1 h at 37˚C, and then 3 μg of protease inhibitor cocktail (Sigma), 8 μg of RNase A (Sigma) and 8 μg of DNase (Qiagen) per ml of pellet were added to the suspension. After 30 min at room temperature, cells were mechanically disrupted by 3 to 4 passages in a SLM Aminco French Pressure cell disrupter, and then centrifuged at 12,000 × g and 4˚C for 10 min to remove unbroken cells. Supernatant was collected and total protein concentration was determined as previously described with the BCA Protein Assay Kit.

Detection of mouse IgG by ELISA

Detection of serum total IgG against the Δ*vraG*Δ*hemB* vaccination strain and each of the bovine IMI isolates was performed to demonstrate and measure the systemic humoral response generated by the immunization of mice. For target antigens, Nunc MaxiSorpTM 96-well plates (Thermo Fisher Scientific Inc., Rochester, NY) were coated with 100 μl of each of the whole *S. aureus* cell extracts or of the recombinant IsdH protein (10 μg/ml diluted in carbonate/bicarbonate buffer, Sigma), and incubated overnight at room temperature. The plates were then saturated with PBS containing 5% skim milk powder for 1 h at 37˚C, followed by a second blocking step with an addition of 5% porcine serum to prevent unspecific *S. aureus* protein A interactions, in the case of whole-cell extracts. One hundred microliters of two-fold serial dilutions of the sera in the dilution buffer (PBS with 2% milk and 0.025% TweenTM 20) were loaded into the plates and incubated for 1 h at 37˚C. Plates were then washed three times with PBS containing 0.05% TweenTM 20, and loaded with 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1/5000 in the dilution buffer. After 1 h of incubation at 37˚C followed by washes, peroxidase activity was detected using 3,3′,5,5′-tetramethylbenzidine (TMB) reagent (KPL Inc., Gaithersburg, MD) according to the manufacturer's recommendations.

Statistical analysis

Statistical analyses were carried out with the GraphPad Prism software (v.6.02). Intracellular bacterial CFUs and bacterial CFUs/g of gland (IMI in mice) were transformed in base 10 logarithm values before being used for statistical analyses. Statistical tests used for the analysis of each experiment and significance are specified in the figure legends.

Results

Validation of the SCV phenotype

Homologous recombination was used to generate *hemB* mutants in the *S. aureus* wild-type and Δ*vraG* isogenic backgrounds. The *hemB* deletion was confirmed by PCR and by

sequencing of the PCR product. The gene *hemB* codes for an δ-aminolevulinate dehydratase, an essential enzyme in porphyrin biosynthesis converting δ-aminolevulnic acid to porphobilinogen [38]. Lacking this enzyme, the *hemB* mutant does not synthesize heme resulting in a defective electron transport system and ATP synthase activity. The *hemB* mutant thus produces much less energy and secondary metabolism is impaired. This phenotypically translates into a slow growth. *In vitro* characterization of mutants confirmed the expected small-colony phenotype of SCVs. After 48 h of incubation at 37˚C on TSA, colonies of *S. aureus* Δ*hemB* and Δ*vraG*Δ*hemB* were approximately 0.5 mm in diameter and appeared non-pigmented, whereas colonies of the parent and Δ*vraG* strains were 4 mm or greater in diameter with a bright yellow pigmentation. The lack of pigmentation in SCVs was previously documented [27]. Growth of the *S. aureus* Δ*hemB* mutants reached a plateau at a lower bacterial density in broth culture compared to wild-type *S. aureus*, but chemical complementation by the addition of hemin (1 μg/ml) in TSB restored the capacity of *S. aureus* Δ*hemB* to reach a bacterial density equivalent to that of the parent strain (data not shown). Similar results were obtained for the Δ*vraG*Δ*hemB* double mutant compared to its isogenic strain Δ*vraG*. Wild-type and Δ*vraG* showed no difference in growth in broth cultures using TSB or milk as cultivation medium, as shown in a previous study [19]. Finally, the ATCC 29213 strain, the single mutants or the double mutant produced equivalent amounts of biofilm compared to that measured for the majority of bovine mastitis isolates studied in a previous study [13].

These results show validation of the SCV phenotypes in *hemB* mutants and demonstrate that chemical complementation by supplemental hemin restores the wild-type phenotype to the full extent.

A mutation in gene *vraG* impairs *S. aureus* internalization in BMECs

We compared the infectivity of the wild-type, Δ*vraG*, Δ*hemB* and Δ*vraG*Δ*hemB* strains in infection and persistence assays using MAC-T cells. By comparing the three mutant strains to their isogenic parent, distinct effects of mutations in gene *hemB* and *vraG* were observed. A short 3-h incubation of bacteria with cell monolayers followed by the addition of lysostaphin to eliminate extracellular bacteria demonstrated good levels of internalization into MAC-T cells for both the wild-type and Δ*hemB* strains, based on the recovery of intracellular CFUs. On the other hand, the single Δ*vraG* mutant showed significantly less ($P \leq 0.01$) internalization compared to its parental strain (Fig 1A). The reduction in internalization as seen with Δ*vraG* was even more pronounced when comparing the double mutant Δ*vraG*Δ*hemB* to Δ*hemB*, with a 10-fold reduction of inoculum recovery in the 3-h internalization assay ($P \leq 0.001$, Fig 1A). This initial reduction of internalized bacterial load was still apparent 12 and 24 h post invasion (PI) for the double mutant strain Δ*vraG*Δ*hemB* (Fig 1B), as illustrated by the 1-log10 reduction of CFU/ml at both time points compared to that observed for Δ*hemB* ($P \leq 0.001$). The difference in initial intracellular bacterial loads between the single Δ*vraG* mutant and wild-type strains (Fig 1A) gradually vanished with longer incubation times (Fig 1B), as both strains did not well persist in MAC-T cells (Fig 2). On the contrary, intracellular CFUs recovered for the single Δ*hemB* strain was significantly higher compared to that recovered for the three other strains at 24 h PI (Fig 1B, $P \leq 0.001$ against all). Globally and as expected for the SCV phenotype, the Δ*hemB* strain showed a better intracellular persistence than any of the other strains over time (Fig 2).

These results suggest that the Δ*vraG* mutation greatly reduces the internalization process into MAC-T cells. Results further demonstrate that the Δ*vraG*Δ*hemB* mutant is still capable of internalization and persistence into BMECs, but to a lesser degree than that seen with the single Δ*hemB* mutant.

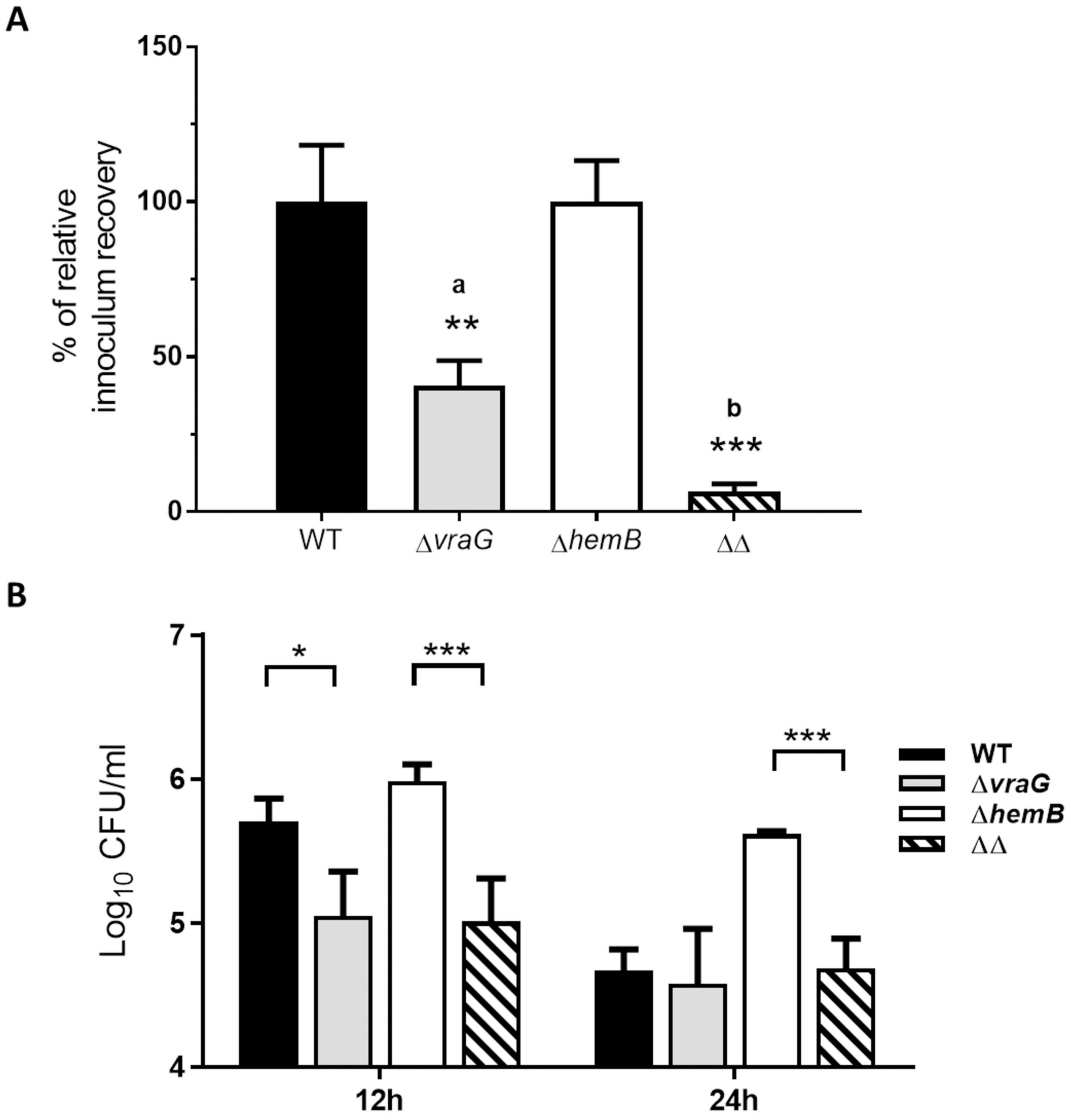

Fig 1. Influence of *S. aureus* Δ*hemB*, Δ*vraG*, and Δ*vraG*Δ*hemB* mutations on MAC-T cell infectivity. MAC-T cells were infected with each of the four strains for 3h, then were incubated with lysostaphin an additional 30 min (t = 3h), 12h or 24h and lysed for measurement of intracellular bacteria (CFUs). (A) Relative recovery of the initial inoculum found within cells at 3h for the Δ*vraG* and Δ*vraG*Δ*hemB* (ΔΔ) mutants. Results are normalized according to that obtained with ATCC 29213 (WT) for comparison to Δ*vraG*, or with Δ*hemB* for comparison to Δ*vraG*Δ*hemB* (ΔΔ), and are expressed as means with SD (**, $P \leq 0.01$; ***, $P \leq 0.001$; unpaired t test). (B) Means and SD of intracellular CFUs for WT and mutants at 12h (left) and 24h (right). A two-way ANOVA and Tukey's multiple comparisons test was used (*: $P \leq 0.05$; ***: $P \leq 0.001$). All values indicate the mean of three independent experiments, each performed in triplicate.

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Δ*vraG*Δ*hemB* and Δ*hemB* SCVs cause low BMEC disruption

As reported above, SCV strains showed a greater persistence over time in MAC-T cells, as illustrated by their sustained intracellular viability at 12 and 24 h PI in comparison to the wild-type and Δ*vraG* strains (Figs 1B and 2). Percent of inoculum recovered from cells stayed nearly the same from 0 to 24 h after lysostaphin addition, both for the double and single *hemB* mutants, with a slight increase at 12 h, indicating intracellular growth (Fig 2). Both strains started to decrease at a slow rate after this time point of 12 h. However, the apparent reduction

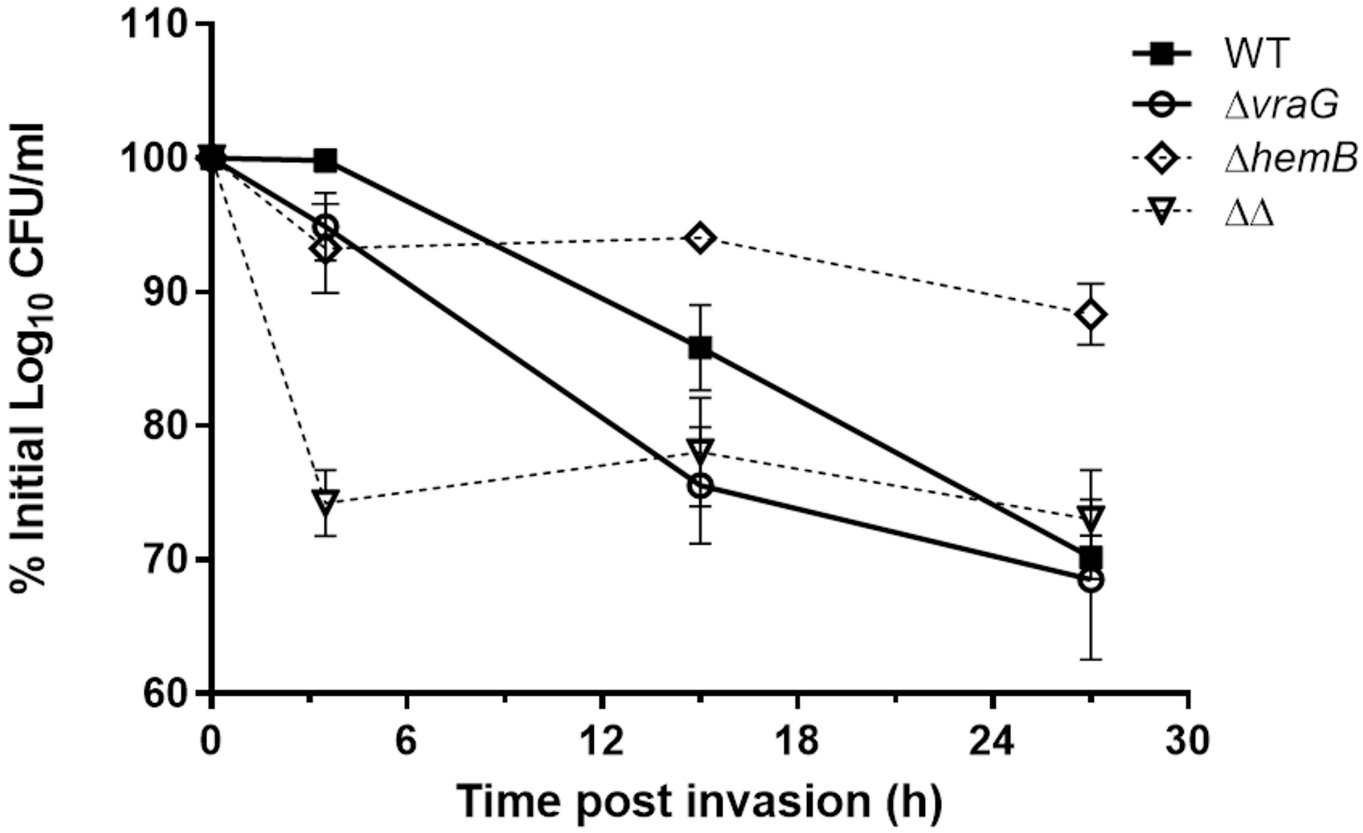

Fig 2. Persistence of *S. aureus* ATCC 29213 (WT) and isogenic mutants within MAC-T cells over time. MAC-T cells were infected with each of the four strains for 3h, then were incubated with lysostaphin an additional 30 min, 12h or 24h and lysed for measurement of intracellular bacteria (CFU). Intracellular bacterial CFUs are expressed as the percentage of the initial inoculum after being transformed in base 10 logarithmic values (Log_{10} CFU/ml). The full lines relate to strains of the normal phenotypes (WT and Δ*vraG*), whereas the dotted lines represent the strains having the SCV phenotype (Δ*hemB* and Δ*vraG*Δ*hemB* [ΔΔ]). Values indicate the mean of three independent experiments, each done in triplicate, with standard deviations.

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of intracellular CFUs for the WT and Δ*vraG* strains was concomitant with the visual observation of increasing damage to cell monolayers over time, in comparison to that observed with strains of the SCV phenotype. This prompted us to evaluate MAC-T cells viability following infection by each of the four strains studied. MAC-T cell viability was evaluated by the MTT method in the exact same conditions that were used for the determination of intracellular bacterial counts.

As expected, both SCV strains caused significantly less MAC-T cytotoxicity in this assay in contrast to that seen with the wild-type and Δ*vraG* strains: when compared to Δ*hemB*, the wild-type strain nearly reduced by half the viability of cells at 12 h (Fig 3A: wild-type, 25.4%; Δ*hemB*, 48.4%). This difference was still apparent at 24 h (Fig 3B: 16.25 *vs*. 34.55%, respectively), even if the bacterial load was 10 times higher for the Δ*hemB* mutant (Fig 1B). The MAC-T cells were more damaged by Δ*hemB* than by the double mutant Δ*vraG*Δ*hemB* but the difference was only significant at 24 h ($P \leq 0.01$). The double mutant sustained epithelial cells viability 2.3 times more than the wild-type strain at 12 h (Fig 3A) and 2.7 times more at 24 h (Fig 3B) ($P \leq 0.0001$ for both time points). Therefore, the greater intracellular persistence of both SCV strains compared to the wild-type and Δ*vraG* strains over time (Fig 2) was likely to be attributed to a lower toxicity of the SCVs for MAC-T cells (Fig 3).

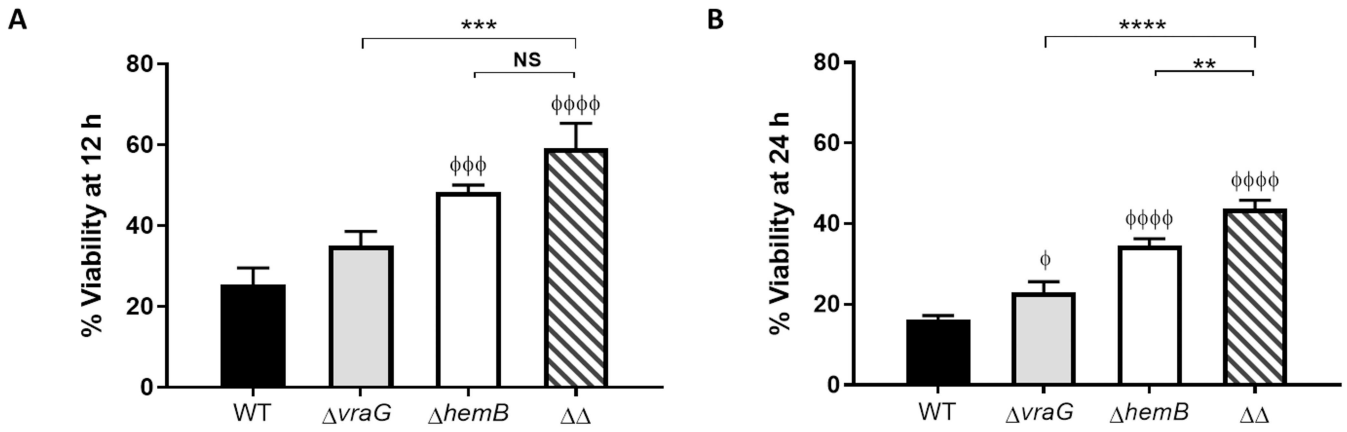

Fig 3. Viability of MAC-T cells infected by *S. aureus* ATCC 29213 (WT) and isogenic mutants. MAC-T cells were infected with each of the four strains (WT, Δ*vraG*, Δ*hemB* and Δ*vraG*,Δ*hemB* [ΔΔ]) for 3h, then were incubated with lysostaphin for 12 h (A) or 24 h (B). MTT viability assays were then performed as described in materials and methods. The results are reported as percent viability relative to uninfected cells and are expressed as the mean with SD of three independent experiments done in triplicate. Statistical significance with the "Φ" symbols are compared to the WT, and the "*" symbols compare the indicated strains (Two-way ANOVA and Tukey's multiple comparisons test: * or Φ, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ΦΦΦΦ, $P \leq 0.0001$).

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Taken together, the results from the BMECs infection assays provide evidence of an additive effect of both Δ*hemB* and Δ*vraG* mutations for the attenuation of the wild-type strain; the *vraG* mutation mainly lowering the intracellular bacterial load and the *hemB* mutation creating the SCV phenotype that increases MAC-T cells viability.

Δ*vraG*Δ*hemB* double mutant is strongly attenuated in a mouse IMI model and is efficiently cleared from mammary glands

To attest the attenuation of Δ*vraG*Δ*hemB* in an *in vivo* model of infection, the virulence of the double mutant was evaluated and compared to the wild-type strain in a murine IMI model. For both strains, the exponential phase of infection took place mainly within the first 12 h post-infection, while the maximal bacterial burden was reached at 24 h for the double mutant and 48 h (day 2 [D2]) for the wild-type strain (Fig 4). At 24 h, the double mutant showed a reduction of 1.9 log10 in mean CFU/g of gland compared to the wild-type ($P \leq 0.05$). Also after 24 h, the mutant bacterial burden showed a constant decline until complete bacterial clearance was reached at day 12 (shown by the asterisk on Fig 4). In contrast, the parental strain provoked severe invasive infections compared to the mutant, killing 3 of the 9 remaining mice at day 2 and 2 of 3 mice at day 7 (Fig 4; arrows) before glands could be harvested for those groups. Mice surviving the WT infection maintained high viable counts (9 log10 CFU/g of gland) at day 7, an approximate 5 log10 difference in bacterial burden compared to the double mutant. These results clearly demonstrate a markedly reduced capacity of the double mutant Δ*vraG*Δ*hemB* to multiply and survive in the mammary gland.

Inflammatory response to Δ*vraG*Δ*hemB* and WT strains following IMI

To monitor the inflammatory response of the mice to infections with the wild-type and mutant strains, neutrophil infiltration in glands was evaluated by the MPO enzymatic activity in gland homogenates. MPO activity in biological samples has previously been correlated with the absolute number of neutrophils [47], and is hence an adequate representation of neutrophil infiltration. During the first hours after infection, neutrophil recruitment followed similar profiles for the double mutant and wild-type infected glands (Fig 5), with exponential intensification

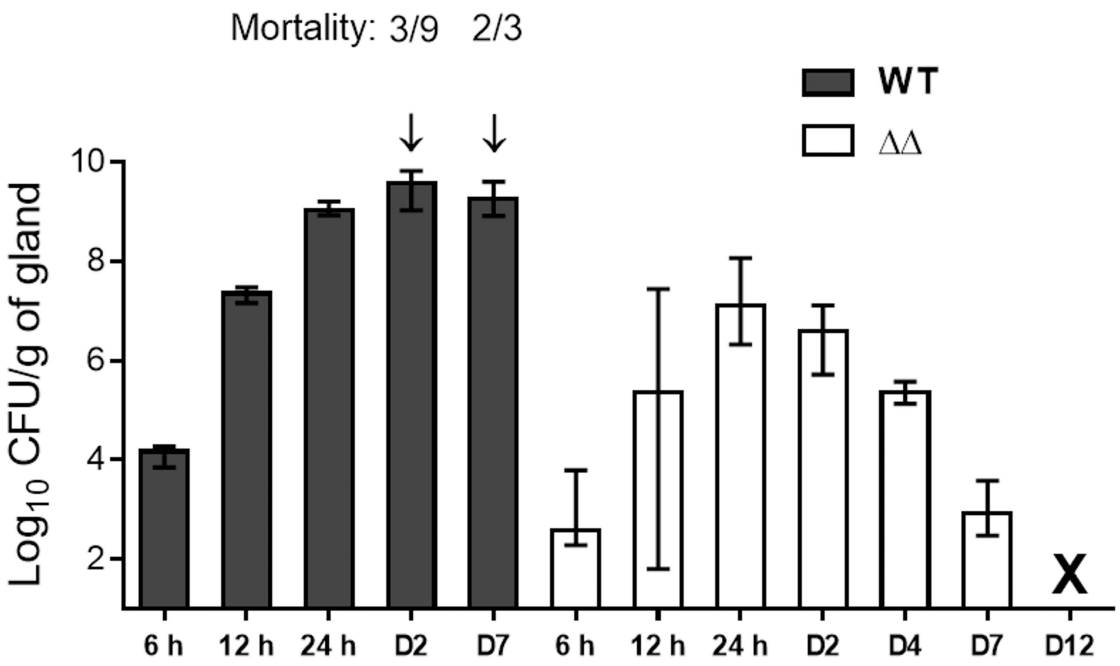

Fig 4. Murine IMIs with the parental (WT) and Δ*vraG*Δ*hemB* (ΔΔ) strains. Mice were infected as previously described and glands were harvested at the indicated hour (h) or day (D) after infection. Each column represents the median value of bacterial CFU counts for a group of glands, and ranges are indicated by bars. A minimum of six glands per group were used except for the WT strain at D7 (2 glands: only one mouse survived). Mortality of mice at specific time points is indicated by arrows. The X indicates the clearance of Δ*vraG*Δ*hemB* from glands (below the detection limit of 10 CFU/gland).

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of apparent neutrophil infiltration from 12 h to 24 h post infection coinciding with bacterial growth albeit with a certain delay. We indeed previously showed that the absolute numbers of polymorphonuclear cells in relation to the bacterial load in mammary glands does not always peak at the same time [48]. No significant difference in MPO activity could be observed at 6, 12 and 24 h between glands infected by mutant and wild-type strains (Fig 5). This equivalence in apparent neutrophil infiltration did not however correlate with the visual observation of inflammation at 24 h, at which point the wild-type infection generated extensive redness of glands in comparison to the double mutant (photographs of Fig 6). In contrast, mutant infected glands were not visually altered at the macroscopic level compared to non-infected controls. The disparity between the visual assessment of inflammation and neutrophil infiltration results could be attributed to the differences in bacterial loads (Fig 4) and the cytotoxic activity of the wild-type strain (Fig 3). Hence, these results indicate that neutrophil recruitment in the glands infected by the double mutant ΔvraGΔ*hemB* strain was equivalent to that seen with the wild-type strain and that this was sufficient to allow a subsequent decline and clearance of the mutant bacterial loads.

The inflammatory response of Δ*vraG*Δ*hemB*-infected glands goes back to normal levels with bacterial clearance

In order to attest strain safety, keeping in mind the possible use of the double mutant as a live-attenuated vaccine, and to confirm that this inflammatory response was not consequent to an inadmissible reactogenic strain, we continued monitoring of MPO activity in Δ*vraG*Δ*hemB*-infected glands 4 and 12 days after infection. The level of MPO activity was then compared to

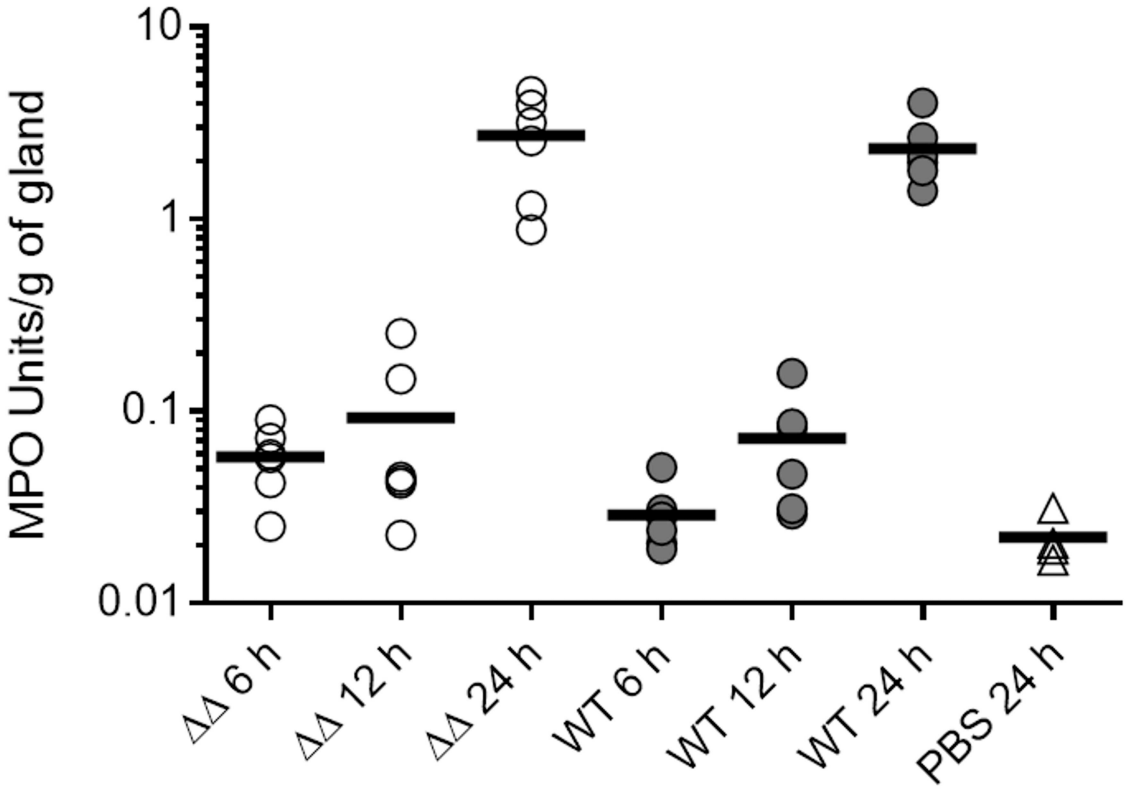

Fig 5. Double mutant Δ*vraG*Δ*hemB* (ΔΔ) stimulates neutrophil influx in mammary glands at levels comparable to *S. aureus* ATCC 29213 (WT) in the first 24 h of infection. Mice were infected as described in materials and methods, and a non-infected control group of mice received a sterile PBS injection (PBS). Glands were harvested at the indicated times, homogenized and kinetically assayed for MPO activity as described in materials and methods. Each dot represents MPO Units for one gland and is shown as a raw value adjusted by gram of gland. Means are represented by thick lines.

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levels obtained for glands from non-infected mice. As illustrated in Fig 7, the apparent neutrophil presence in mutant infected glands was still high 4 days after infection, with MPO activity ranging from 8 to 21 Units/g of gland. The levels of MPO at this time point might be the direct consequence of the mammary gland involution, the process by which the lactating gland returns to a morphologically near pre-pregnant state. Indeed, involution is normally associated with neutrophil recruitment allowing phagocytosis of apoptotic cells during the remodelling of tissue [49]. However, later on, the MPO levels in the mutant infected glands went through a substantial decline between days 4 and 12, ($P \leq 0.01$). MPO concentration was then considered to be back to normal levels at day 12, showing no significant difference with the non-infected glands.

Immunizations with Δ*vraG*Δ*hemB* generate a strong humoral response against several *S. aureus* bovine IMI isolates

To confirm that immunization with the attenuated strain Δ*vraG*Δ*hemB* can indeed generate a strong immune response suitable for its use as a putative live vaccine against *S. aureus* IMIs, mice were immunized with different doses of the mutant and serum total IgGs were assayed by ELISA for detection of antigenic components present in whole-cell extracts of a variety of *S. aureus* bovine isolates. A specific detection of the staphylococcal iron-regulated IsdH protein was also attempted by ELISA. Doses of 10^6, 10^7 and 10^8 CFUs, when administered

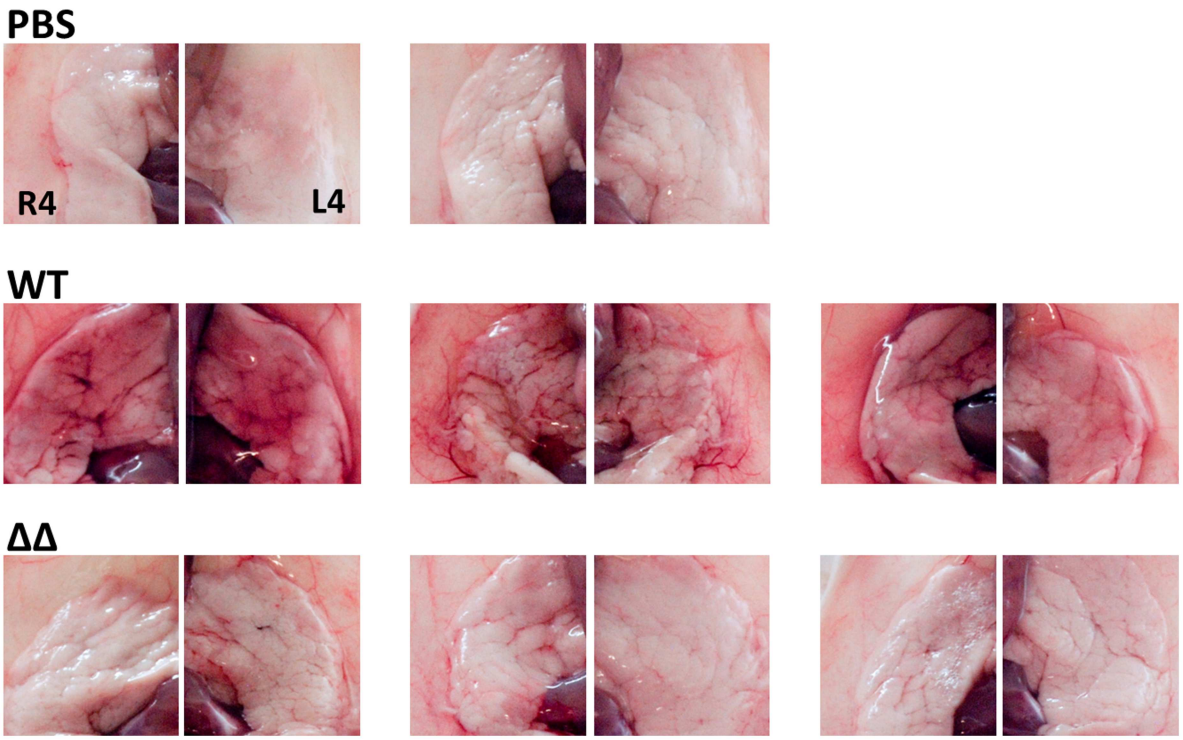

Fig 6. Visual inflammation of the large R4 and L4 mouse mammary glands 24 h after IMI with *S. aureus* ATCC 29213 (WT) and the double mutant Δ*vraG*Δ*hemB* (ΔΔ). Mice were infected as described in materials and methods, and the non-infected control group of mice received a sterile PBS injection (PBS). Pictures show the pairs of glands (R4, left, and L4, right) for each mouse in each group (PBS, n = 2 mice; WT, n = 3 mice; ΔΔ, n = 3 mice) that were harvested after 24 h.

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subcutaneously in the neck, triggered no adverse effect such as modification of mice behavior, signs of inflammation, or necrosis at the immunization site throughout the immunization period. Additionally, immunizations using increasing quantities of the live double mutant Δ*vraG*Δ*hemB* yielded increasing titers of systemic IgG antibodies against its own whole cell extract (Fig 8A). The titers of the immune sera were significantly higher than those of the pre-immune sera, demonstrating specificity of antibody production against the *S. aureus* antigens present in the live vaccine. Most importantly, increasing the doses of Δ*vra*GΔ*hemB* also generated a consequential rise of antibody titers against a variety *S. aureus* strains isolated from bovine mastitis, including strains from the major *spa* types found in Canada and elsewhere in the world (Fig 8B). Interestingly, it was also possible to generate specific IgGs against the cell wall-associated and iron-regulated protein IsdH as demonstrated in the ELISA using this protein as the antigen (Fig 8C). These results clearly show that (i) immunization with the double mutant can raise a specific immune response against *S. aureus*, and that (ii) the strain background (ATCC 29213) share sufficient common features with bovine mastitis strains so that the antibody response also strongly recognizes strains of major *spa* types. Additionally, the presence of IgG2a and IgG1 isotypes specific to IsdH, *i.e.*, indicative of a Th1 and Th2 oriented immune response, respectively, was assayed for serums collected from mice immunized with the double mutant and compared to that obtained from mice immunized with the purifed IsdH protein. Significantly higher IgG2a/IgG1 titer ratios ($P \leq 0.05$) were found for serums from mice immunized with the live-attenuated double mutant compared to the ratios obtained from mice vaccinated with the purified IsdH protein (Fig 8D).

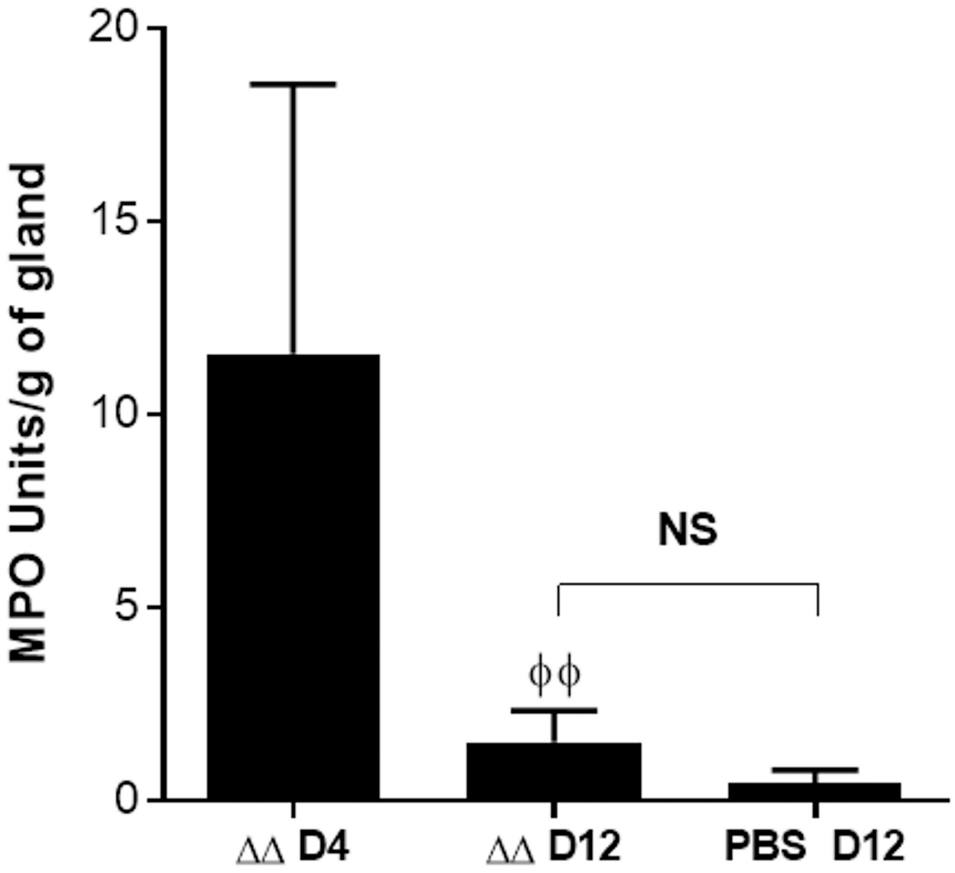

Fig 7. Neutrophil infiltration goes back to normal levels after clearance of the double mutant Δ*vraG*Δ*hemB* (ΔΔ) from the mammary glands. Mice were infected as described in materials and methods, and a non-infected control group of mice received a sterile PBS injection (PBS). Glands were harvested at the indicated times, homogenized and kinetically assayed for MPO activity as described in materials and methods. Columns represent means of MPO Units of a group of 6 glands (4 for the PBS control) adjusted by gram of gland, and error bars illustrate standard deviation. Statistical significance between the Day 4 and 12 groups post infection is shown by the "Φ" symbols. One-Way ANOVA and Tukey's multiple comparison tests were used (ΦΦ, $P \leq 0.01$; NS, no significant difference between groups).

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Discussion

The ability of *Staphylococcus aureus* to express multiple virulence factors permitting host colonization, tissue destruction, immune evasion, intracellular persistence and biofilm production makes it a very challenging pathogen to fight. Vaccines designed to prevent IMI in bovine mastitis therefore have to take into account the complexity of *S. aureus* pathogenesis as well as the diversity of strains capable of causing mastitis including strains with the SCV phenotype. SCVs are known to be somewhat attenuated but have intracellular abilities that allow persistence in the host without producing invasive infections [24]. In this study, we further attenuated the SCV phenotype to demonstrate that this phenotype could be used as a live attenuated vaccine.

One of our recent research endeavors has been to identify genes that are highly expressed by multiple *S. aureus* strains *in vivo*. The proteins encoded by these genes represent good targets as vaccination agents or in drug development as they are more likely to have an importance in virulence and, being expressed, to be efficiently targeted by the immune response. In a previous study, we used a DNA microarray approach to uncover *S. aureus* genes that were

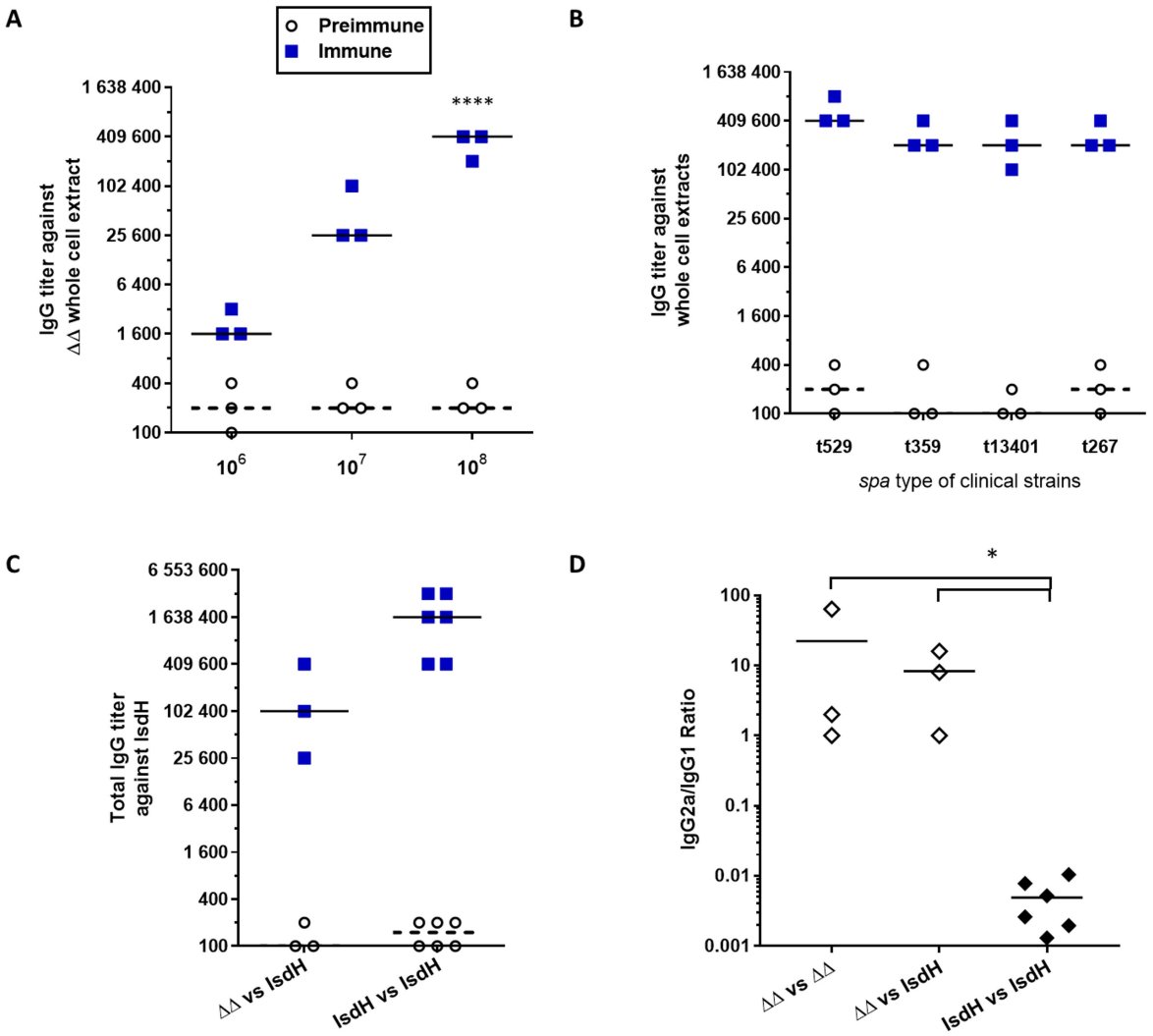

Fig 8. Immunization of mice with the live-attenuated double mutant (Δ720Δ*hemB*) induces a strong humoral response against *S. aureus* bovine mastitis isolates and against a specific cell-wall associated antigen (IsdH). Mice were immunized as previously described: serums were collected before priming immunization (preimmune, open circles) and ten days after the boost immunization (immune, blue squares). **A.** IgG titers rise with increasing immunization doses (10^6, 10^7, 10^8 CFU) of the live-attenuated mutant Δ*vraG*Δ*hemB*: each dot represents the total IgG titer of one mouse against a Δ*vraG*Δ*hemB* whole cell extract. Medians are represented by thick lines for immune titers and dashed lines for preimmune titers. Titers were compared to their corresponding preimmune titers (Two-way ANOVA and Tukey's multiple comparisons test: ****: $P \leq 0.0001$). **B**. Immunization with the live-attenuated mutant Δ*vraG*Δ*hemB* confers high IgG titers against components that are shared by mastitis strains of commonly found *spa* types. Each dot represents the total IgG titer of one mouse against the whole cell extract of the indicated strain. Medians are represented by thick lines for immune titers and dashed lines for preimmune titers. All immune titers were compared to their corresponding preimmune titers (Two-way ANOVA and Tukey's multiple comparisons test: $P \leq 0.0001$ for all groups). **C.** Immunization with the live-attenuated mutant Δ*vraG*Δ*hemB* confers specific IgG titers against the cell-wall associated protein IsdH. Each dot represents the total IgG titer of one mouse against recombinant IsdH. Compared groups were immunized with the 10^8 CFU of the live-attenuated Δ*vraG*Δ*hemB* (ΔΔ) or 25 μg of the purified recombinant IsdH protein (IsdH). **D.** IgG isotype ratios (IgG2a/IgG1) of mice immunized with the live-attenuated mutant Δ*vraG*Δ*hemB* (open diamonds) or immunized with the recombinant IsdH (black diamonds), against whole-cell extracts of strain Δ*vraG*Δ*hemB* (vs ΔΔ) or against the recombinant IsdH protein (vs IsdH). Each diamond represents the IgG2a/IgG1 titer ratio for one mouse. Medians are represented by thick lines (One-way ANOVA and Dunn's multiple comparison test: *: $P \leq 0.05$).

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highly expressed by several strains in experimentally induced bovine IMIs [19]. The *S. aureus* operon *vraFG* (SACOL0718-720) was among several genes strongly expressed by *S. aureus* in the mammary gland environment [19].

The operon *vraFG* codes for an ABC transporter-like system with a role in resistance to antibiotics [32,50–52] and to several cationic antimicrobial peptides (CAMPs) such as indolicidin isolated from bovine neutrophils [30,53], human cathelicidin LL-37 [54] and Class I bacteriocins such as nisin A and nukacin ISK-1 [55]. Noteworthy, *vraFG* was shown not only to be under the regulation of the two-component regulatory system *graXRS*, but also to play an essential role by sensing the presence of CAMPs and signaling through *graS* to activate *graR*-dependent transcription, including its own transcript [30]. Besides, *vraFG* does not act as a detoxification module as previously believed [32], as it cannot confer resistance when produced on its own [30]. It was also reported that the expression of two key determinants, *mprF* and *dlt*, (needed for the modification of bacterial surface charged residues) is dependent upon *graXRS-vraFG*, and that these effectors are responsible for making the surface charge globally less negative [32], thus promoting resistance. When the sensing system or its effectors are altered, an increased susceptibility to vancomycin [32], daptomycin, polymyxin B [52] and several host defense CAMPs [56] is observed.

Our previous studies revealed that gene *vraFG* (SACOL0718-720) was up-regulated in both fresh milk *in vitro* and in milk recovered from infected cows. But of greater significance, this gene was shown to be a key factor in *S. aureus* virulence in cows, since a Δ*vraG* mutant was greatly attenuated in experimental bovine IMIs [19]. Consequently, this mutation was selected to further attenuate the SCV phenotype by generating the double mutant (Δ*vraG*Δ*hemB*) investigated in the present work.

This study is the first one, to our knowledge, to consider the use of classical respiratory deficient SCVs as the foundation of a non-virulent, genetically-defined attenuated vaccine for the delivery of *S. aureus* antigens. Live-attenuated strains of *S. aureus* have been of great interest for a long time and have been studied for immunization of cows since the '80s [57]. Some teams have managed to produce attenuation by chemical mutagenesis [58] in order to elicit high specific humoral response in cows, but unfortunately this caused only a weak reduction in shedding of bacteria, and no difference in the reduction of somatic cell counts (SCC) in milk when vaccinated groups were challenged. Besides, the genetic basis for the attenuation of this strain was still unknown, which may be a concern considering the necessity to obtain a stable and safe vaccine. In a different manner, transposon mutagenesis was used to generate an aromatic amino acid auxotrophic *aroA* mutant of *S. aureus* for testing in a mouse IMI model [59]. Both Th1 and Th2 responses were elicited, and a certain degree of protection was observed against homologous and heterologous *S. aureus*. The mutant was also demonstrated safe in leukopenic mice in a model of nasal colonization [60], but its immunogenicity in cows remains unknown.

In this study, genetic stabilization of the SCV phenotype (*i.e.*, the *hemB* deletion) along with inactivation of an important effector of the resistance to cationic compounds (*i.e.*, the *vraG* deletion) were able to generate an attenuated *S. aureus* strain that still exhibited a low transient internalization in epithelial cells. Since SCVs are expected to show a high capacity of invasion and intracellular persistence [23], the reduction we observed in post-invasion intracellular bacterial loads was attributed to the disruption of gene *vraG*. Since inappropriately high intracellular invasion and persistence might not be suited for a strain intended to be used as a live vaccine (even if low internalization in cells might help stimulating cell-mediated immunity), this second mutation was considered relevant for attenuation, especially in the SCV background.

More specifically; the lesser degree of internalization and intracellular persistence in BMECs observed for Δ*vraG* and especially for Δ*vraG*Δ*hemB*, as well as the total clearance of the latter mutant from glands in mice, suggested an additive deleterious effect of the two mutations. Because of their reduced membrane potential (ΔΨ), respiratory deficient SCVs (having an altered electron transport chain) are generally expected to be more resistant to cationic compounds or antibiotics that require membrane polarization for their mode of action [27,61–62]. However, other unknown mechanisms and factors can also lead to a decreased [63], or even a higher susceptibility of SCVs to such compounds, as previously shown with the frog-derived CAMP dermaseptin [64]. Also, electron transport SCVs have been shown to be more susceptible to oxidant damage caused to their membrane, because of their limited ability to generate a ΔΨ [65]. Therefore, it is likely that disruption of the *graXRS-vraFG* regulon via *vraG* mutation in the SCV background (*i.e.*, Δ*vraG*Δ*hemB*) may be more deleterious than that seen with the normal phenotype (*i.e.*, Δ*vraG*) because of the lack of membrane potential, which is required for active detoxification and reactive oxygen species (ROS) protection [66].

Another explanation for the strong attenuation seen for the double mutant is the possibility that *graXRS* and *vraFG* act as key regulators in the stress response of SCVs. The alternative transcription factor sigma B (SigB) is known to affect the expression of several genes encoding virulence factors and stress-response systems specific to SCVs [21]. This regulator has a permanent activity in *hemB* mutants [67] and was shown to play a role in biofilm production and in the intracellular persistence of SCVs [21]. VraFG may act in concert with SigB constant influence or possibly through another mechanism involving PhoU. PhoU is a global negative regulator of genes involved in central carbon metabolism and cytochrome expression and is therefore connected to the SCV phenotype [61]. In *S. aureus*, PhoU is important for resistance to CAMPs [68] and has been shown to regulate *dlt*, which is also under the control of *graXRS-vraFG*. Besides, GraSR has been linked to virulence and stress response pathways, which could help the SCV and normal phenotypes to persist in the host environment [69].

The low expression of invasive virulence factors such as hemolysin-α and other toxins associated with the reduced quorum-sensing activity of SCVs [39], probably resulted in the relatively low BMEC cytotoxicity of SCVs observed in this study. Nevertheless, the precise mechanisms by which non-SCV *S. aureus* strains kill epithelial cells are not completely understood and could be attributed to both induction of apoptotic pathways and/or pore-forming related lysis [23,27]. One of the prominent results of this study hinges on the high attenuation of virulence that was attained with the double mutant in the mouse IMI model. The parental strain was highly virulent and resulted in considerable mortality in this model, whereas a 5-log10 reduction in CFU/g of gland followed by total bacterial clearance from the glands was observed for the double mutant. The double mutant strain showed a good capacity to stimulate the recruitment of neutrophils in the gland and most importantly, this inflammatory response was not associated with tissue damage. Histopathological examinations of inoculated glands in future investigations will help to further support innocuity at the microscopic level.

This pro-inflammatory response was a first clear indicator of the potential of the double mutant strain as a live-attenuated vaccine. When administered through the subcutaneous route, the marked attenuation of the double mutant permitted the use of relatively high doses of live bacteria to immunize mice, without provoking any sign of local inflammation or adverse effect. At the same time, this immunization allowed to trigger a broad systemic response that translated in high IgG titers against whole *S. aureus* cell components. This humoral response was also broad enough to react against several bovine mastitis isolates represented by the most prevalent *S. aureus spa* types found in Canadian dairy herds [13] and elsewhere in the world [34]. Furthermore, the response was found to include significant IgG titers against the staphylococcal iron-regulated and cell-wall associated IsdH antigen. IgG isotypes

produced against this antigen also allowed to demonstrate a more balanced Th1 and Th2 response as compared to that obtained when immunizing with the purified IsdH antigen. This feature might help to improve protection against *S. aureus*, for which control is increasingly thought to require cell-mediated immunity [15–18]. Noteworthy, although this proof of concept demonstrated that the double mutant genetic background (ATCC 29213) share many common features with bovine mastitis strains, such mutations (*i.e.*, Δ*vraG*Δ*hemB*) and attenuation can be created in any desired background if one wishes to cover specific types of strains. The demonstration of protection elicited by such a vaccine against experimental IMI in mice, and then in cows, will need to be examined in future work, along with investigations on the best possible route of administration. The use of cows is clearly important for future studies; nevertheless, we have recently shown that results from our mouse model of IMI [70] can translate very well to that obtained in cows [71].

As a final note, the administration route of such vaccines might undeniably influence the qualitative properties of immune response and efficacy of protection. On this matter, it was previously reported that intramammary but not intraperitoneal application of live temperature-sensitive *S. aureus* could stimulate murine mucosal responses against a challenge with a homologous virulent strain [72]. A different study was conducted by using formalin-killed whole cells of planktonic and biofilm *S. aureus* to immunize mice [73]. It was shown that the biofilm vaccine performed better in immunogenicity and protection when administered by the intramammary route, despite the fact that the planktonic subcutaneous vaccine triggered a significantly higher humoral response. In more recent work, the same team reported that subcutaneous immunizations with staphylococcal protein A could elicit higher humoral responses against the antigen, but that the response was more balanced (humoral and cellular) when administered by intramammary injections [74]. However, this subunit vaccine failed to protect immunized mice challenged (IMI) with a strong biofilm-producing and encapsulated *S. aureus* strain, regardless of the route of immunization. In this manner, the route of administration of our genetically defined live-attenuated vaccine will definitively impact the level of its protective efficacy, and additional practical aspects will need to be considered (*e.g.*, subcutaneous administration *vs*. intramammary perfusion into four quarters for a whole herd) in upcoming studies.

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Project administration: FM.

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Supervision: FM.

Writing – original draft: JCG.

Writing – review & editing: JCG BGT FM.

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ANNEXE III

WO2018072031 - VACCINE CONSTRUCTS AND USES THEREOF AGAINST STAPHYLOCOCCUS INFECTIONS

Présentation du brevet

Cette application pour un brevet, "VACCINE CONSTRUCTS AND USES THEREOF AGAINST STAPHYLOCOCCUS INFECTIONS", a été déposée puis publiée (26/04/2018) suite aux travaux de notre équipe ayant mené à l'identification des gènes fortement exprimés lors de la mammite par *Staphylococcus aureus*, puis à ceux ayant démontré que les produits de ces gènes peuvent être utilisés comme formulation vaccinale sous la forme de fusions protéiques ou peptidiques et/ou en combinaison avec une souche atténuée SCV de *S. aureus* pour améliorer la réponse immune contre ces produits. L'**Annexe III** présente une partie des déclarations d'inventions, soient celles discutant de la portée des inventions, du contexte et du sommaire de celles-ci. En résumé, nous avons protégé a) des polypeptides de fusion présentant une immunogénicité accrue et leur utilisation comme vaccin contre les IIMs staphylococciques; b) l'utilisation de souches vivantes atténuées de *S. aureus* basées sur les aspects phénotypiques des SCVs à des fins vaccinales pour fournir une réponse immunitaire contre ces souches et augmenter l'efficacité protectrice du vaccin. La description plus détaillée des différents items protégés est précisée dans la section *summary of the invention* de l'annexe.

Les inventeurs sont : François Malouin, Céline Ster, **Julie Côté-Gravel** et Eric Brouillette.
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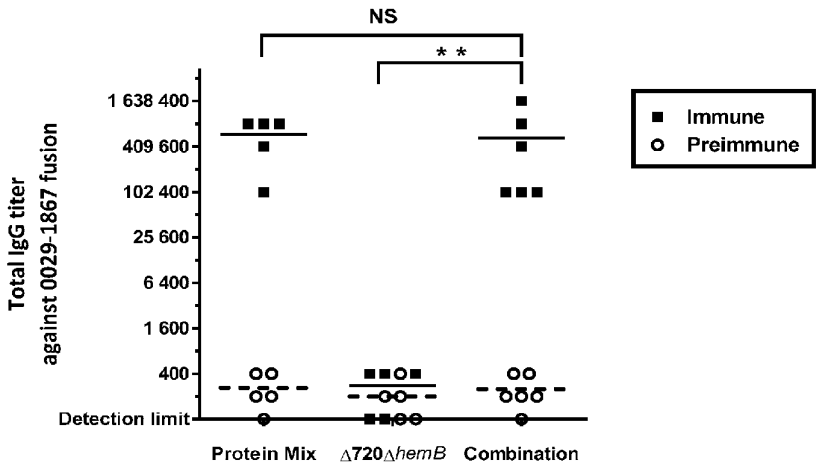

FIG. 16

(57) Abstract: There is provided a fusion construct of formula (I): X-A-linker-B-Z (I) wherein : (1) A and B are identical or different and are independently: (a) a polypeptide comprising a SACOL0029 polypeptide as set forth in any one of the sequences depicted in FIG. 24 (SEQ ID NOs: 5 and 121 to 131), a SACOL0264 polypeptide (SEQ ID NO : 185), a SACOL0442 polypeptide as set forth in any one of the sequences depicted in FIG. 22D (SEQ ID NOs: 29 and 82 to 92), a SACOL0718 polypeptide (SEQ ID NO : 186), a SACOL0720 polypeptide as set forth in any one of the sequences depicted in FIGs. 23I-J (SEQ ID NOs: 11 and 109 to 120), a SACOL1353 polypeptide (SEQ ID NO : 187), a SACOL1416 polypeptide (SEQ ID NO : 188), a SACOL1611 polypeptide (SEQ ID NO : 189), a SACOL1867 polypeptide as set forth in any one of the sequences depicted in FIG. 25D (SEQ ID NOs: 152 to 164), a SACOL1912 polypeptide (SEQ ID NO : 43), a SACOL1944 polypeptide (SEQ ID NO : 190), a SACOL2144 polypeptide (SEQ ID NO : 191), a SACOL2365 polypeptide (SEQ ID NO : 192), a SACOL2385 polypeptide (SEQ ID NO : 50) or a SACOL2599 polypeptide (SEQ ID NO : 193), based on the gene nomenclature from the Staphylococcus aureus COL (SACOL) genome set forth in

WO 2018/072031 A1

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CLAIMS

1. A fusion construct of formula (I):

X-A-linker-B-Z (I)

wherein :

(1) A and B are identical or different and are independently:

(a) a polypeptide comprising a SACOL0029 polypeptide as set forth in any one of the sequences depicted in FIG. 24 (SEQ ID NOs: 5 and 121 to 131), a SACOL0264 polypeptide (SEQ ID NO: 185), a SACOL0442 polypeptide as set forth in any one of the sequences depicted in FIG. 22D (SEQ ID NOs: 29 and 82 to 92), a SACOL0718 polypeptide (SEQ ID NO: 186), a SACOL0720 polypeptide as set forth in any one of the sequences depicted in FIGs. 23I-K (SEQ ID NOs: 11 and 109 to 120), a SACOL1353 polypeptide (SEQ ID NO : 187), a SACOL1416 polypeptide (SEQ ID NO : 188), a SACOL1611 polypeptide (SEQ ID NO : 189), a SACOL1867 polypeptide as set forth in any one of the sequences depicted in FIG. 25D (SEQ ID NOs: 152 to 164), a SACOL1912 polypeptide (SEQ ID NO : 43), a SACOL1944 polypeptide (SEQ ID NO : 190), a SACOL2144 polypeptide (SEQ ID NO : 191), a SACOL2365 polypeptide (SEQ ID NO : 192), a SACOL2385 polypeptide (SEQ ID NO : 50) or a SACOL2599 polypeptide (SEQ ID NO : 193), based on the gene nomenclature from the *Staphylococcus aureus* COL (SACOL) genome set forth in NCBI Reference Sequence NC_002951.2;

(b) a polypeptide encoded by a gene from a same operon as a gene encoding the polypeptide of (a);

(c) a polypeptide comprising an immunogenic fragment of at least 13 consecutive amino acids of (a) or (b);

(d) a polypeptide comprising an amino acid sequence at least 60% identical overall to the sequence of the polypeptide of any one of (a) to (c); or

(e) a polypeptide comprising an immunogenic variant comprising at least 13 consecutive amino acids of any one of (a) to (c);

(2) the linker is an amino acid sequence of at least one amino acid or is absent;

(3) X is absent or is an amino acid sequence of at least one amino acid; and

(4) Z is absent or is an amino acid sequence of at least one amino acid.

2. The construct of claim 1, wherein (1) (a) is a polypeptide comprising a SACOL0029 polypeptide as set forth in any one of the sequences depicted in FIG. 24 (SEQ ID NOs: 5 and 121 to 131), a SACOL0442 polypeptide as set forth in any one of the sequences depicted in FIG. 22D (SEQ ID NOs: 29 and 82 to 92), a SACOL0720 polypeptide as set forth in any one of the sequences depicted in FIGs. 23I-K (SEQ ID NOs: 11 and 109 to

120), or a SACOL1867 polypeptide as set forth in any one of the sequences depicted in FIG. 25D (SEQ ID NOs: 152 to 164).

3. The construct of claim 2, wherein at least one of A and B is (a) a polypeptide comprising a SACOL0029 polypeptide as set forth in any one of the sequences depicted in FIG. 24 (SEQ ID NOs: 5 and 121 to 131); (b) a polypeptide encoded by a gene from a same operon as a gene encoding the polypeptide of (a); (c) a polypeptide comprising an immunogenic fragment of at least 13 consecutive amino acids of (a) or (b); (d) a polypeptide comprising an amino acid sequence at least 60% identical overall to the sequence of the polypeptide of any one of (a) to (c); or (e) a polypeptide comprising an immunogenic variant comprising at least 13 consecutive amino acids of any one of (a) to (c); and the other one of A and B is (a') a polypeptide comprising a SACOL1867 polypeptide as set forth in any one of the sequences depicted in FIG. 25D (SEQ ID NOs: 152 to 164); (b') a polypeptide encoded by a gene from a same operon as a gene encoding the polypeptide of (a'); (c') a polypeptide comprising an immunogenic fragment of at least 13 consecutive amino acids of (a') or (b'); (d') a polypeptide comprising an amino acid sequence at least 60% identical overall to the sequence of the polypeptide of any one of (a') to (c'); or (e') a polypeptide comprising an immunogenic variant comprising at least 12 consecutive amino acids of any one of (a') to (c').

4. The construct of claim 2, wherein at least one of A and B is (a) a polypeptide comprising a SACOL0442 polypeptide as set forth in any one of the sequences depicted in FIG. 22D (SEQ ID NOs: 29 and 82 to 92); (b) a polypeptide encoded by a gene from a same operon as a gene encoding the polypeptide of (a); (c) a polypeptide comprising an immunogenic fragment of at least 13 consecutive amino acids of (a) or (b); (d) a polypeptide comprising an amino acid sequence at least 60% identical overall to the sequence of the polypeptide of any one of (a) to (c); or (e) a polypeptide comprising an immunogenic variant comprising at least 13 consecutive amino acids of any one of (a) to (d); and the other one of A and B is (a') a polypeptide comprising a SACOL0720 polypeptide as set forth in any one of the sequences depicted in FIGs. 23I-K (SEQ ID NOs: 11 and 109 to 120); (b') a polypeptide encoded by a gene from a same operon as a gene encoding the polypeptide of (a'); (c') a polypeptide comprising an immunogenic fragment of at least 13 consecutive amino acids of (a') or (b'); (d') a polypeptide comprising an amino acid sequence at least 60% identical overall to the sequence of the polypeptide of any one of (a') to (c'); or (e') a polypeptide comprising an immunogenic variant comprising at least 12 consecutive amino acids of any one of (a') to (d').

5. The construct of claim 2, wherein A and B are identical or different and are (a) a polypeptide comprising a SACOL0720 polypeptide as set forth in any one of the sequences depicted in FIGs. 23I-K (SEQ ID NOs: 11 and 109 to 120); (b) a polypeptide encoded by a gene from a same operon as a gene encoding the polypeptide of (a); (c) a polypeptide comprising an immunogenic fragment of at least 13 consecutive amino acids of (a) or (b); (d) a polypeptide comprising an amino acid sequence at least 60% identical overall to the sequence of the polypeptide of any one of (a) to (c); or (e) a polypeptide comprising an immunogenic variant comprising at least 13 consecutive amino acids of any one of (a) to (d).

6. The construct of any one of claims 1-2 and 4, wherein said immunogenic fragment (d) comprises one or more of the following amino acid sequences: KDTINGKSNKSRNW (SEQ ID NO: 34); and KDGGKYTLESHKELQ

(SEQ ID NO: 1).

7. The construct of claim 6, wherein said immunogenic fragment (d) comprises one or more of the following amino acid sequences : STQNSSSVQDKQLQKVEEVPNNSEKALVKKLYDRYSKDTINGKSNKSRNWVYSERPLNENQVRIHLEGTYTV AGRVYTPKRNITLNKEVVTLKELDHIIRFAHISYGLYMGEHLPKGNIVINTKDGGKYTLESHKELQKDRENVKINT ADIKNVTFKLVKSVNDIEQV (SEQ ID NO: 30); DKQLQKVEEVPNNSEKALVKKLYDRYSKDTINGKSNKSRNWVYSERPLNENQVRIHLEGTYTVAGRVYTPKR NITLNKEVVTLKELDHIIRFAHISYGLYMGEHLPKGNIVINTK (SEQ ID NO :32); and DKQLQKVEEVPNNSEKALVKKLYDRYSKDTINGKSNKSRNWVYSERPLNENQVRIHLEGTYTVAGRVYTPKR NITLNKEVVTLKELDHIIRFAHISYGLYMGEHLPKGNIVINTKDGGKYTLESHKELQKDRENVKINTADIKNVTFKL VKSVNDIEQV (SEQ ID NO : 33).

8. The construct of any one of claims 1-2 and 4-5, wherein said immunogenic fragment (d) comprises one or more of the following amino acid sequences: QFGFDLKHKKDALA (SEQ ID NO: 21); TIKDQQKANQLAS (SEQ ID NO: 22); KDINKIYFMTDVDL (SEQ ID NO: 23); and DVDLGGPTFVLND (SEQ ID NO: 24).

9. The construct of claim 8, wherein said immunogenic fragment (d) comprises one or more of the following amino acid sequences: RASLSSEIKYTAPHDVTIKDQQKANQLASELNNQKIPHFYNYKEVIHTKLYKDNLFDVKAKEPYNVTITSDKYIP NTDLKRGQADLFVAEGSIKDLVKHKKHGKAIIGTKKHHVNIKLRKDINKIYFMTDVDLGGPTFVLNDKDYQEIRK YTKAKHIVSQFGFDLKHKKDALALEKAKNKVDKSIETRSEAISSISSLTG (SEQ ID NO : 12); ASLSSEIKYTAPHDVTIKDQQKANQLASELNNQKIPHFYNYKEVIHTKLYKDNLFDVKAKEPYNVTITSDKYIPNT DLKRGQADLFVAEGSIKDLVKHKKHGKAIIGTKKHHVNIKLRKDINKIYFMTDVDLGGPTFVLNDKDYQEIRKYT KAKHIVSQFGFDLKHKKDALALEKAKNKVDKSIETRSEAISSISSLTG (SEQ ID NO: 13); ASLSSEIKYTAPHDVTIKDQQKANQLASELNNQKIPHFYNYKEVIHTKLYKDNLFDVKAKEPYNVTITSDKYIPNT DLKRGQADLFVAEGSIKDLVKHKKHGKAIIGTKKHHVNIKLRKDINKIYFMTDVDLGGPTFVLNDKDYQE (SEQ ID NO: 14); KDINKIYFMTDVDLGGPTFVLNDKDYQEIRKYTKAKHIVSQFGFDLKHKKDALA (SEQ ID NO : 15); KDINKIYFMTDVDLGGPTFVLNDKDY (SEQ ID NO : 17); KDINKIYFMTDVDLGGPTFVLNDKD (SEQ ID NO : 16); KDINKIYFMTDVDLGGPTFVLND (SEQ ID NO : 19); KHIVSQFGFDLKHKKDALA (SEQ ID NO: 20) and SQFGFDLKHKKDALA (SEQ ID NO: 18).

10. The construct of any one of claims 1-3, wherein said immunogenic fragment (c') comprises one or more of the following amino acid sequences: PYNGVVSFKDATGF (SEQ ID NO: 165); AHPNGDKGNGGIYK (SEQ ID NO: 167); SISDYPGDEDISVM (SEQ ID NO: 169); RGPKGFNFNENVQA (SEQ ID NO: 172); QFESTGTIKRIKDN (SEQ ID NO: 175); and GNSGSPVLNSNNEV (SEQ ID NO: 178).

11. The construct of claim 10, wherein said immunogenic fragment (d) comprises the following amino acid sequence: TQVKDTNIFPYNGVVSFKDATGFVIGKNTIITNKHVSKDYKVGDRITAHPNGDKGNGGIYKIKSISDYPGDEDIS VMNIEEQAVERGPKGFNFNENVQAFNFAKDAKVDDKIKVIGYPLPAQNSFKQFESTGTIKRIKDNILNFDAYIEP

GNSGSPVLNSNNEVIGVVYGGIGKIGSEYNGAVYFTPQIKDFIQKHIEQ (SEQ ID NO: 39).

12. The construct of any one of claims 1 to 11, wherein the linker comprises at least four identical or different amino acids selected from the group consisting of glycine, serine, alanine, aspartate, glutamate and lysine.

13. The construct of any one of claims 1 to 12, wherein the linker comprises (GGGGS)n (SEQ ID NO: 67), (ERKYK)n (SEQ ID NO: 61); or (EAAAK)n (SEQ ID NO: 63), wherein n=1 to 5.

14. The construct of any one of claims 1 to 13, wherein said X comprises a polyhistidine of 6 to 10 amino acids.

15. The construct of any one of claims 1 to 13, wherein said X is absent.

16. The construct of any one of claims 1 to 15, wherein said Z is absent.

17. An isolated nucleic acid molecule encoding the construct defined in any one of claims 1 to 16.

18. A vector comprising the isolated nucleic acid defined in claim 17.

19. A host cell comprising the vector defined in claim 18.

20. The cell of claim 19, which is a live attenuated form of *Staphylococcus aureus*.

21. The cell of claim 20, wherein the live attenuated form of *Staphylococcus aureus* has a stabilized small colony variant (SCV) phenotype.

22. The cell of claim 21, wherein the live attenuated form of *Staphylococcus aureus* having a stabilized SCV phenotype is a Δ*hemB*Δ720 *S. aureus*.

23. A composition comprising:

(A) at least one of the constructs defined in any one of claims 1 to 16; at least one of the nucleic acid molecules defined in claim 17; at least one of the vectors defined in claims 18; or at least one of the cells defined in any one of claims 19 to 22; and

(B) (i) a polypeptide defined in any one of claims 1 to 11;

(ii) a live attenuated *Staphylococcus aureus*;

(iii) a pharmaceutically acceptable excipient;

(iv) an adjuvant; or

(v) a combination of at least two of (i) to (iv).

24. The composition of claim 23, wherein the live attenuated form of *Staphylococcus aureus* expresses:

(a) a polypeptide comprising a SACOL0029 polypeptide as set forth in any one of the sequences depicted in FIG. 24 (SEQ ID NOs: 5 and 121 to 131), a SACOL0264 polypeptide (SEQ ID NO: 185), a SACOL0442 polypeptide as set forth in any one of the sequences depicted in FIG. 22D (SEQ ID NOs: 29 and 82 to 92), a SACOL0718 polypeptide (SEQ ID NO: 186), a SACOL0720 polypeptide as set forth in any one of the sequences depicted in FIGs. 23I-J (SEQ ID NOs: 11 and 109 to 120), a

SACOL1353 polypeptide (SEQ ID NO : 187), a SACOL1416 polypeptide (SEQ ID NO : 188), SACOL1611 (SEQ ID NO : 189), a SACOL1867 polypeptide as set forth in any one of the sequences depicted in FIG. 25D (SEQ ID NOs: 152 to 164), a SACOL1912 polypeptide (SEQ ID NO : 43), SACOL1944 (SEQ ID NO : 190), a SACOL2144 polypeptide (SEQ ID NO : 191), a SACOL2365 polypeptide (SEQ ID NO : 192), a SACOL2385 polypeptide (SEQ ID NO : 50) or a SACOL2599 polypeptide (SEQ ID NO : 193) based on the gene nomenclature from the *Staphylococcus aureus* COL (SACOL) genome set forth in NCBI Reference Sequence NC_002951.2;

(b) a polypeptide encoded by a gene from a same operon as a gene encoding the polypeptide of (a);

(c) a polypeptide comprising an immunogenic fragment of at least 13 consecutive amino acids of (a) or (b);

(d) a polypeptide comprising an amino acid sequence at least 60% identical overall to the sequence of the polypeptide of any one of (a) to (c); or

(e) a polypeptide comprising an immunogenic variant comprising at least 13 consecutive amino acids of any one of (a) to (c).

25. The composition of claim 23 or 24, wherein the live attenuated form of *Staphylococcus aureus* has a stabilized small colony variant (SCV) phenotype.

26. The composition of any one of claims 23 to 25, wherein the adjuvant comprises alum, an oil, saponin, cyclic-diguanosine-5'-monophosphate (c-di-GMP), polyphosphasine, indolicidin, pathogen-associated molecular patterns (PAMPS), liposome or a combination of at least two thereof.

27. A method for preventing and/or treating a Staphylococcal intramammary infection (IMI) in a mammal, said method comprising administrating to said mammal an effective amount of the construct defined in any one of claims 1 to 16; of the nucleic acid molecule defined in claim 17; of the vector defined in claim 18; of the cell defined in any one of claims 19 to 22; or of the composition defined in any one of claims 23 to 26.

28. The method of claim 27, wherein said Staphylococcal IMI is caused by one or more *Staphylococcus aureus* strains.

29. The method of claim 27 or 28, wherein said mammal is a cow.

30. A use of an effective amount of (i) the construct defined in any one of claims 1 to 16; (ii) the nucleic acid molecule defined in claim 17; (iii) the vector defined in claim 18; of the cell defined in any one of claims 19 to 22; (iv) the composition defined in any one of claims 23 to 26; or (v) a combination of at least two of (i) to (iv), for preventing and/or treating a Staphylococcal intramammary infection (IMI) in a mammal.

31. The use of claim 30, wherein said Staphylococcal IMI is caused by one or more *Staphylococcus aureus* strains.

32. The use of claim 30 or 31, wherein said mammal is a cow.

33. The construct defined in any one of claims 1 to 16; the nucleic acid molecule defined in claim 17; the vector

defined in claim 18; the cell defined in any one of claims 19 to 22; the composition defined in any one of claims 23 to 26 or a combination of at least two thereof, for use in the prevention and/or treatment of a Staphylococcal intramammary infection (IMI) in a mammal.

34. The construct, nucleic acid molecule, vector, cell, composition or combination of claim 33, wherein said Staphylococcal IMI is caused by one or more *Staphylococcus aureus* strains.

35. The construct, nucleic acid molecule, vector, cell or composition of claim 33 or 34, wherein said mammal is a cow.

36. A kit for preventing and/or treating a Staphylococcal intramammary infection (IMI) in a mammal comprising:

(A) (i) at least one of the constructs defined in any one of claims 1 to 16; (ii) at least one of the nucleic acid molecules defined in claim 17; (iii) at least one of the vectors defined in claim 18; (iv) at least one of the cells defined in any one of claims 19 to 22; or (v) a combination of at least two of (i) to (iv), and

(B) (i) the polypeptide defined in any one of claims 1 to 11;

(ii) a live attenuated *Staphylococcus aureus*;

(iii) a pharmaceutically acceptable excipient;

(iv) an adjuvant;

(v) instructions for using the kit for preventing and/or treating a Staphylococcal intramammary infection (IMI) in a mammal; or

(vi) a combination of at least two of (i) to (v).

ANNEXE IV

IMMUNE AND EXPERIMENTAL INFECTION RESPONSES OF DAIRY COWS VACCINATED WITH THE COMBINATION OF SIX *STAPHYLOCOCCUS AUREUS* PROTEINS THAT ARE EXPRESSED DURING BOVINE INTRAMAMMARY INFECTION AND A TRIPLE ADJUVANT

Introduction de l'article et contribution des auteurs

Cette étude correspond à l'évaluation du vaccin sous-unitaire contre la mammite bovine mis au point par notre équipe à l'Université de Sherbrooke, ayant mené à une entente de commercialisation par la pharmaceutique Bayer animal Heath. L'équipe a mis au point un vaccin basé sur six des antigènes de la virulence de *S. aureus* surexprimés pendant l'infection chez le bovin laitier, couplé à un triple adjuvant, et procédé à l'immunisation et le *challenge* d'un groupe de vaches laitières pour le comparer à un groupe de vaches placebo. La caractérisation des réponses immunes des vaches ainsi que de leur réponse au *challenge* expérimental sont ainsi présentés. Les auteurs ont contribué à cette publication de la façon suivante : la conception du projet de recherche fut réalisée par Céline Ster, François Malouin et Pierre Lacasse. Céline Ster, Marianne Allard, Simon Boulanger et moi-même avons effectué les expériences. L'article a été écrit par Céline Ster, François Malouin et Pierre Lacasse

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Immune and experimental infection responses of dairy cows vaccinated with the combination of six *Staphylococcus aureus* proteins that are expressed during bovine intramammary infection and a triple adjuvant

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Abstract

Staphylococcus aureus is a leading cause of bovine intramammary infections (IMI). Standard antibiotic treatments are not very effective and currently available vaccines lack tangible efficacy. Developing a vaccine formulation for *S. aureus* mastitis is challenging and selection of target antigens is critical. The gene products of six *S. aureus* genes that are highly expressed during IMI were selected as antigens for this study. The vaccine contained six recombinant proteins formulated with Emulsigen®-D, a CpG oligodeoxynucleotide and indolicidin. Nine cows in mid-lactation received the vaccine while ten received saline (placebo). Two immunizations were performed 10 weeks apart. All the antigens induced an immune response. A balanced immune response (IgG2/IgG1 ratio of 1) was observed for antigen SACOL0442 while a predominant Th2 response was observed for the other antigens (IgG2/IgG1 ratio <1). Immunizations induced CD4+ cell proliferation in response to SACOL0442, SACOL0029, SACOL0720 and SACOL1912 while a CD8+ cell proliferation was induced by SACOL0720. Four weeks after the second immunization, three quarters per animal were experimentally infused with ~60 CFU of *S. aureus*. Although no difference in *S. aureus* counts was observed between the two groups after this robust infectious challenge, a sustained reduction in milk somatic cells counts (SCC) was observed in vaccinated cows. A correlation between SCC and *S. aureus* counts in milk was also observed. Altogether, this indicates that the collective immune responses induced by the antigens certainly contribute to the observed benefits of the whole vaccine. More work is needed to understand how different antigens stimulate a different response using the same adjuvant.

Keywords: *Staphylococcus aureus*, bovine mastitis, immunization, *in-vivo*-expressed antigen, challenge, subunit vaccine

1. Introduction

Bovine intramammary infections (IMI) are one of the major diseases affecting dairy cows worldwide. In Canada, *Staphylococcus aureus* is the most frequent pathogen isolated from clinical IMI and the second most frequent microorganism causing subclinical IMI (Reyher et al., 2011). These infections decrease milk quality and are a reservoir for transmission within the herd. Presently, antibiotherapy is the method of choice to treat IMI although the cure rate can vary from 4 to 92 % for subclinical *S. aureus* IMI (Barkema et al., 2006). The cost of IMI is very high for the dairy producers. It was evaluated at 662 CA$ per lactating cow per year for the Canadian dairy farms (Aghamohammadi et al., 2018). This underlines the need for other methods to control *S. aureus* and vaccination may represent a key strategy in the struggle against bovine IMI.

Although two commercials inactivated whole cell vaccines are currently available (Lysigin®, Boehringer Ibgelhiem Vetmedica, MO and StartVac®, Hippra, Spain), their efficacy remains controversial (Landin et al., 2015; Middleton et al., 2006; Nickerson et al., 2008; Schukken et al., 2014). As pointed out by Middleton (2008), the low efficacy of vaccines tested so far may be due to the lack of expression of the targeted proteins during IMI. In a previous study by our group, a series of genes that were highly expressed by *S. aureus* during IMI were identified. The high and sustained levels of *in vivo* expression of these genes suggested that they play an important role for maintenance of the bacteria during infection and suggested that immune responses that target these antigens may neutralize *S. aureus*. Among these highly expressed genes, six were selected as vaccine targets for this study. SACOL0442 is annotated as a *S. aureus* enterotoxin and thus possibly interfere with the immune response as a superantigen (Tuffs et al., 2018). SACOL0720,

also known as VraG, is the receptor of an ABC transporter (VraFG) which is implicated in resistance against cationic antimicrobial peptides (CAMPs) that are part of the innate immune system but which also trigger specific immune responses (Tomasinsig et al., 2010). The peptide loop of SACOL0720 that was selected as the antigen for this study was also described as being implicated in the detection of CAMPs (Falord et al., 2012). A *S. aureus* VraG mutant was also evaluated as a possible live-attenuated vaccine for the treatment of IMI (Côté-Gravel et al., 2016). When compared to its bacterin (heat killed), this live VraG mutant improved humoral and cellular immune responses against *S. aureus* in mice (Côté-Gravel et al., 2019). Furthermore, mutants of either SACOL442 or SACOL720 were found to be greatly attenuated in their ability to colonize the cow mammary gland (Allard et al., 2013). SACOL1867 is one of *S. aureus* serine proteases (Spl) that are likely involved in host-pathogen interactions to promote *S. aureus* dissemination (Paharik et al., 2016). Besides, little is known on the importance of antigens SACOL0029, SACOL1912 or SACOL2385 in *S. aureus* virulence although both SACOL1912 (Csb33) and SACOL2385 (Hsp20) are stress-related proteins with Csb33 being part of the SigB regulon (Gertz et al., 2000) and Hsp20 being one of the molecular chaperones (Henderson et al., 2006), respectively. Altogether, these six antigens were selected because they were expressed by multiple *S. aureus* strains at multiple time points during experimental IMI of multiple cows (Allard et al., 2013). Selection was also based on the predicted localization of the protein (surface of the bacterium or extracellular) and/or on diversity of functions (known or putative).

Adjuvants are also an important component of vaccines. Many commercial adjuvants were developed in the last fifteen years for veterinarian purposes. They could limit the local reaction at the injection site and trigger the appropriate immune response more efficiently than conventional

adjuvants like alum (Young, 2019). While alum permits delivery of the antigen, oil-in-water emulsions (such as Emulsigen®D, MPV) can also activate the immune system and double emulsions (water-in-oil-in-water, such as Montanide ISA 201, Seppic) have the propriety to target the antigen presenting cells (APC). Also, immunostimulants such as CpG can improve the activation of the immune system and target APC. Ioannou et al. (2002) have shown that the addition of CpG-containing oligodeoxynucleotides to Emulsigen®-D can improve the magnitude of the immune response and can direct the response towards the production of IgG2 instead of IgG1. More and more molecules are described as immunostimulants such as indolicidin that was first defined as an antimicrobial agent (CAMP). Kovacs-Nolan et al. (2009) showed that the use of a triple adjuvant (CpG, indolicidin and polyphosphazene) increased the retention of the antigen at the injection site and induced a strong antigen-specific Th1 biased immune response in cattle. Fine-tuning of the adjuvant could thus promote a high-magnitude and long-lasting immune response and help attaining the desired type of response (the production IgG1 or IgG2 or the activation of T cells) (Mutwiri et al., 2011) Although much progress has been made in the development of adjuvants for bovines, the efficacy of the vaccine still depends on the antigens used, the route of immunization and the schedule of vaccination.

The aims of the present study are to evaluate the cellular and humoral immune responses induced by vaccination using antigens expressed by *S. aureus* during IMI combined with a triple adjuvant, and to evaluate the protective effect of this vaccine against experimentally induced *S. aureus* IMI in dairy cows.

2. Materials and methods

2.1. Production of the antigens

The antigens selected for the vaccine are encoded by genes that are highly expressed during *S. aureus* bovine IMI (Allard et al., 2013): SACOL0029, SACOL0442, SACOL0720, SACOL1867, SACOL1912, SACOL2385 (Table 1). His-tagged recombinant proteins SACOL0442 and SACOL0720 were designed and produced using QIA expression technology (pQE30 plasmid) from Qiagen Inc. (Mississauga, ON, Canada), according to the manufacturer's recommendations. The other His-tagged proteins were designed and produced by GenScript Inc (Piscataway, NJ).

2.2. Dairy cows

Nineteen healthy multiparous Holstein cows in mid-lactation were selected for this project and randomly assigned to the vaccinated group (n=9) or the placebo group (n=10). Parity average was 4.22 ±1.30 for the vaccine group and 3.5 ± 1.58 for the placebo group. Days in milk average at the beginning of the project was 140.22 ± 40.50 for the vaccinated cows and 150.40 ± 60.14 for the placebo cows. Milk production average at the beginning of the project was 26.88 ± 5.44 kg/day for the vaccinated cows and 26.95 ± 5.75 kg/day for the placebo cows. All selected cows started the project with SCC below 150 000 cells/mL (5.17 log10). They also were all selected for the absence of *S. aureus* IMI in their four quarters. During the immunization period, SCC were monitored every 4 weeks and microbiological analysis were performed four and ten weeks after the beginning of the immunization program. The cows were housed in a biosafety level 2 barn at Sherbrooke Research and Development Centre of Agriculture and Agri-Food Canada (Sherbrooke, QC).

2.3. Immunization of dairy cows

The vaccine was composed of 300 µg of each of the six antigens combined with Emulsigen®-D (30 % v/v, oil-in-water adjuvant, MVP Technologies, Omaha, NE), 1 mg CpG ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT, kindly provided by VIDO-InterVac, Saskatoon, SW) and 4 mg of the cationic peptide indolicidin (ILPWKWPWWPWRR, Chemprep Inc., Miami, FL). Two immunizations were performed 10 weeks apart, by subcutaneous injections (2 mL per dose) of the formulated vaccine in the neck. In previous studies, we observed that the duration of the primary humoral response to immunization in dairy cows is 8 to 10 weeks (Ster et al., 2010). No systemic adverse side effect of the immunizations was observed. Local swelling was observed at the injection sites, especially after the second immunization. Blood from the caudal vein and milk samples were taken before the first immunization and four weeks after the second immunization for the serum and six weeks after the second immunization (10 days after the beginning of the challenge) for milk for the detection of total IgG, IgG1 and IgG2. Larger volumes of blood from the jugular vein (150 mL) were taken before and 14 weeks after the first immunization (*i.e.*, 4 weeks after the second immunization, just before the beginning of the experimental infection) for peripheral blood mononuclear cells (PBMCs) isolation and analysis of the cellular immune response.

2.4. Detection of total IgG, IgG1 and IgG2 by ELISA

Detection of total IgG, IgG1 and IgG2 against each of the 6 antigens in serum and milk was performed as previously described (Ster et al., 2010) with some modifications. The secondary antibodies used were a horseradish peroxidase (HRP) conjugated goat anti-bovine IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), a HRP conjugated sheep anti-bovine IgG1

(Bio-Rad antibodies, Hercules, CA) or a HRP conjugated sheep anti-bovine IgG2 (Bio-Rad antibodies), diluted 1:50 000 1:20 000 and 1:20 000 respectively in PBS containing 0.5% gelatin (BD, Franklin Lakes, NJ) and 0.1% Tween®20 (Sigma Aldrich, Oakville, ON). Detection of total IgG, IgG1 and IgG2 in skimmed milk (composite sample from all four quarters) was carried out using the same procedure with few modifications. Milk samples were diluted into PBS containing 0.5% gelatin. The sheep anti-bovine IgG2 was diluted 1:10 000 into PBS containing 0.5% gelatin and 0.1% Tween®20. The four borders of the plate were used as negative control and for the calculation of the threshold (average of the OD in the wells plus twice the SEM). Preimmune and immune serums from a cow were always tested in the same plate and titers were determined at least twice. Titers were defined as the inverse of the highest dilution above threshold.

2.5. Evaluation of the cellular immune response

PBMCs were isolated from jugular vein blood and labelled with carboxyfluoroscein diacetate, succinimidyl ester (CFDA-SE; Molecular Probes Inc., Eugene, OR) as previously described (Loiselle et al., 2009). At the end of the CFDA-SE labelling procedure, the PBMCs were suspended in RPMI medium containing 5% FBS and 1X antibiotic/antimycotic agents (A5955, Sigma Aldrich). The PBMCs (5×10^6 cells per well) were stimulated with the mitogen ConA (positive control; Sigma Aldrich) at a final concentration of 1 µg/mL or one of the antigens (5 µg per well) and incubated for 7 d at 37°C with 5% CO_2. As a negative control, the PBMCs were incubated without any stimulant molecules. Stimulations were performed in duplicate (Ster et al., 2010).

The proliferation of CD4+ and CD8+ cells was evaluated after the incubation with the different stimulant molecules. The cells were centrifuged at 300 × *g* for 5 min, suspended in PBS

containing 0.5% BSA (BioShop Canada, Burlington, ON). The mouse anti-bovine CD8 coupled with Alexa Fluor®647 (diluted 1:20, Bio-Rad antibodies) and the mouse anti-bovine CD4 coupled with rPE (diluted 1:20, Bio-Rad antibodies) were then added. After 20 min of incubation on ice, the cells were washed three times with PBS containing 0.5% BSA. The cells were then suspended in PBS with 0.5% formaldehyde. The percentages of the proliferative populations were determined by flow cytometry on a BD FACS Canto II flow cytometer using the BD FACS Diva software.

2.6. Experimentally induced *S. aureus* intramammary infection (challenge)

The *S. aureus* bovine mastitis strain CLJ08-3, previously characterized in our laboratory, was used for the experimental infection (Allard et al., 2013). This strain, like most *S. aureus*, possesses the genes for all antigens present in the vaccine. The day of the challenge, a volume of the overnight culture of *S. aureus* in Mueller Hinton broth (MHB; BD) was transferred to 200 mL of fresh MHB at a concentration of A_{600nm} of 0.1 and subsequently grown at 35°C to obtain 10^8 CFU/mL in the exponential phase of growth. For intramammary infusions, bacteria were diluted in sterile apyrogen PBS (Sigma Aldrich) using sterile apyrogen plasticware. The inoculum was plated on TSA and was found to contain 63 CFU in 3 mL.

Somatic cell count (SCC) determinations and bacterial analysis of aseptic quarter milk samples were carried out prior to experimental IMI to ensure that all cows were free of IMI. Experimental infusion of mammary quarters with bacteria was performed in three (randomly chosen) of the four quarters of each cow after the evening milking according to a procedure previously described with few modifications (Petitclerc et al., 2007). Briefly, teats were first scrubbed with gauze soaked in 70% ethanol. Teats were allowed to air-dry before intramammary infusion of 3 mL of bacterial suspension (containing 63 CFU) into each three of the four quarters. Immediately after infusion,

all quarters were thoroughly massaged and teats were dipped in an iodophore-based teat sanitizer. Disposable gloves were worn throughout the procedure and disinfected before proceeding to the next animal.

2.7. Evaluation of the *S. aureus* viable counts after experimental infection

Aseptic milk samples were taken before the morning milking seven (d-7) and four (d-4) days prior to the experimental infection, the morning of the experimental infection (d1) and then three times a week during the 3 first weeks following the experimental infection and twice a week for the 2 remaining weeks. After foremilk was discarded and the teats were disinfected with 70% ethanol, a 10-mL milk sample was aseptically collected in a 50-mL sterile vial for each individual quarter. Milk samples were 10-fold serially diluted and 100 µL of each dilution were plated on both tryptic soy agar (BD) and mannitol salt agar plates (BD) for CFU determinations and *S. aureus* identification, respectively. Plates were then incubated for 24h at 35°C before the colonies were counted. The dilutions that showed between 30 and 300 colonies were used to calculate the bacterial concentration. Each dilution was plated in duplicate. Bacteriological cure was defined as no bacteria detected (detection limit of 10 CFU/mL) in two consecutive samplings and until the end of the challenge period.

2.8. Evaluation of the somatic cell counts (SCC) and milk production

At the same frequency as for aseptic milk samplings, milk was harvested using individual quarter milking units at morning milking and weighed for the determination of quarter milk production. A non-aseptic 50-mL sample was also taken from each quarter milking units for the determination of the SCC by a commercial laboratory (Lactanet, Ste-Anne-de-Bellevue, QC, Canada). The

milking units were thoroughly washed and disinfected with an iodine-based germicide detergent (K.O. Dyne®, GEA Farm Technologies, Westmoreland, NY) between cows. All other materials in contact with milk were disinfected with 70% ethanol.

2.9. Statistical analysis

Statistical analyses of the experimental infection data was performed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) as repeated measurements. For parameters measured at quarter level, quarter(cow) was used as the subject, while cow was used as subject for the other parameters). For the analysis of SCC, CFU and IgG titers, data were log10 transformed prior to analysis. For the analysis of quarter milk production during the experimental infection, the milk production prior to the experimental infection (measured at d-7 d-4 and d1) was used as covariable. For correlation analysis over the challenge period, means of CFU and CCS were calculated for the 3 infected quarters over the whole period for the vaccinated cows. Means of the different titers at week 14 (just prior the beginning of the challenge) for the vaccinated cows was also performed. Then, all means were log10 transformed and Spearman analysis was performed using SAS.

2.10. Ethics statement

All animal experiments were approved by the Sherbrooke Research and Development Centre animal care committee, protocol number 385, and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

3. Results

3.1. Antibody responses to vaccination

The presence of serum total IgG against each antigen was evaluated prior to the vaccination (preimmune) for the vaccinated and placebo cows. No differences were observed between the 2 groups for the antigens SACOL0029, SACOL0442, SACOL0720, SACOL1912 and SACOL2385. However, for antigen SACOL1867, preimmune titers were higher for the placebo cows than for the vaccinated cows (average titers of 6 560 *vs* 2 555, respectively, $P<0.05$). This results was due to one of the placebo cows which preimmune titer was the highest of all cows (titers of 25 600 whereas the titers ranged from 12 800 to 800 for all other cows). Immunization induced an increase in the serum total antigen-specific IgG for the vaccinated group in comparison to the placebo group (Figure 1A, $P<0.0001$ all antigens considered). In comparison to the preimmune titers, the average two-fold increase of titer for the vaccinated cows was 6.44 ± 1.59 for SACOL1912, 5.67 ± 1.66 for SACOL0720, 5.44 ± 1.94 for SACOL1867, 4.22 ± 1.48 for SACOL2385, 3.11 ± 1.27 for SACOL0442 and 2.67 ± 1.22 for SACOL0029.

No difference in the titers of preimmune serum IgG1 was observed between the vaccinated and placebo groups for all the antigens. Immunization induced an increased production of serum antigen-specific IgG1 for the vaccinated group in comparison to the placebo group (Figure 1B, $P<0.0001$ all antigens considered). In comparison to the preimmune titers, the average two-fold increase of titers for the vaccinated cows was 6.11 ± 0.93 for SACOL1912, 5.67 ± 1.80 for SACOL1867, 5.11 ± 0.93 for SACOL0720, 4.44 ± 1.59 for SACOL2385, 3.78 ± 1.86 for SACOL0029 and 3.22 ± 1.20 for SACOL0442.

Prior to the first vaccination, no difference in the IgG2 preimmune serum titers was observed for any of the antigens. Immunization induced an increased production of serum antigen-specific IgG2 for the vaccinated group which was greater than for the placebo group (Figure 1C, $P<0.0001$ all antigens considered). In comparison to the preimmune titers, the average two-fold

increase of titers for vaccinated cows was 3.78 ± 0.83 for SACOL1867, 3.22 ± 1.09 for SACOL1912, 2.56 ± 0.88 for SACOL0442, 2.11 ± 1.05 for SACOL0720, 1.56 ± 1.33 for SACOL2385 and 1.11 ± 1.05 for SACOL0029.

The milk total IgG, IgG1 and IgG2 titers were also evaluated against all antigens six weeks after the second immunization (corresponding to 10 days after the beginning of the experimental infection, Table 2). Overall, the milk total IgG and IgG1 titers were higher for the vaccinated cows than for the placebo cows ($P<0.0001$ and $P<0.0001$ for total IgG and IgG1, respectively).When antigens are considered individually, milk total IgG titers were higher for the vaccinated cows than for the placebo cows except for SACOL0442 and SACOL1867. Milk IgG1 titers were higher for vaccinated cows for all antigens except for SACOL0442. Milk IgG2 titers were not different for the vaccinated or the placebo group.

To further characterize the antibody response, the serum IgG2/IgG1 ratio was calculated for cows of the vaccinated group, 4 weeks after the second immunization (Figure 2). Interestingly, the IgG2/IgG1 ratio median was 1 for SACOL0442. For the antigens SACOL0029, SACOL0720, SACOL1912 and SACOL2385, the IgG2/IgG1 ratio median was lower than 1 ($P<0.0001$, $P<0.0001$, $P<0.001$, $P<0.001$ and $P<0.0001$, for SACOL0029, SACAOL0720, SACOL1867, SACOL1867, SACOL1912 and SACOL2385, respectively)

3.2 Cellular immune response to the vaccination

The proliferation of CD4+ cells collected before the first immunization was not different between vaccinated cows and placebo cows, except for the stimulation with the antigen SACOL0720 that led to a slightly higher proliferation of CD4+ cells for vaccinated cows *vs* placebo cows (4.85 % *vs* 0.07 %, respectively, $P<0.05$). The proliferation of CD4+ cells was

evaluated again 4 weeks after the second immunization and, proliferation was greater after stimulation with the antigens SACOL0029, SACOL0442, SACOL1912 and SACOL0720 for cells isolated from vaccinated cows compared to that observed for cells isolated from placebo cows (Figure 3A, $P<0.05$). Seven of the nine vaccinated cows responded to all antigen stimulations, while one cow did not respond to the stimulation with SACOL0029 and SACOL0442 and a second one did not respond to the stimulation to SACOL0029, SACOL1867 or SACOL2385.

The preimmune proliferation of CD8+ cells was not different for vaccinated and placebo cows before the first immunization for all antigens. Four weeks after the second immunization, CD8+ cell proliferation was greater after stimulation with the antigen SACOL0720 for vaccinated cows compared to that observed for cells of placebo cows (Figure 3B, $P<0.05$). Six out of nine of the vaccinated cows responded to all antigen stimulations, while one cow did not respond to any of them (although it responded to the positive control ConA), one cow only responded to the antigen SACOL0442 and a third cow did not respond to the stimulation with SACOL0029, SACOL1867 and SACOL2385. The two cows with a partial CD4+ response were the same that showed the weakest CD8+ response.

3.4. Effect of the vaccination on experimentally induced *S. aureus* infection

Prior to the experimental infection, quarter SCC and milk bacteriology were performed on d-7, d-4 and d1. No *S. aureus* IMI was detected and SCC were 4.77 ± 0.05 log10 cells /mL and 4.78 ± 0.06 log10 cells /mL at d-7, 4.72 ± 0.05 log10 cells /mL and 4.79 ± 0.06 cells /mL at d-4 and 4.80 ± 0.08 log10 cells /mL and 4.72 ± 0.06 log 10 cells /mL at d1 for the vaccinated cows and the placebo cows, respectively. Within the five first days after experimental infection of the quarters, four cows (three from the placebo group and one from the vaccinated group) showed body

temperature above 40°C. Over the same period, four cows (all from the placebo group) showed marked modifications in the appearance of the milk (watery appearance, presence of viscous filaments, presence of blood). At d3, *S. aureus* was detected in the milk of 53 of the 57 inoculated quarters. Quarter milk appearance, quarter milk production and body temperature of the two groups during the experimental infection are presented in Table 3. Over the challenge period, vaccination did not reduce *S. aureus* CFU in milk (P=0.29, Figure 4A). However, 3 of the 27 infected quarters of the vaccinated cows underwent bacteriological cure while none of the quarters of the placebo cows (0/30) were cured. Due to the small sample size this was not considered a significant effect.

Vaccination lead to lower milk SCC for the vaccinated cows than for the placebo cows over the challenge period (Figure 4B, P<0.001). Milk SCC were also positively correlated to *S. aureus* CFU over the challenge period (r=0.58, P<0.01). Bacterial counts over the challenge period for vaccinated cows were negatively correlated to the serum total IgG titers against SACOL0720 (r=-0.76, P<0.05) and also negatively correlated to the serum IgG2 titers against SACOL1912 (r=-0.67, P<0.05) measured immediately prior to the experimental infection (corresponding also to 4 weeks after the second immunization).

Quarter milk production was not different between the two groups prior to the experimental infection (3.59 ± 0.20 kg and 3.63 ± 0.19 kg at d-7 (P=0.89), 3.60 ± 0.20, 3.64 ± 0.19 kg at d-4 (P=0.89) and 2.91 ± 0.20 kg and 3.24 ±0.19 kg at d1 (P=0.23) for the vaccinated and placebo cows, respectively). An interaction day×treatment was observed for the milk production of vaccinated quarters (P=0.03). Five days after *S. aureus* infusion, milk production of infected quarters was slightly higher for vaccinated cows than for placebo cows (3.10 kg ± 0.14 and 2.38 kg ± 0.13, respectively, P<0.001, Table 3).

4. Discussion

Because of the limited efficacy of antibiotic treatment, vaccines still appear as the best alternative to hopefully prevent *S. aureus* IMI. Thus far, commercially available vaccines do not protect the animals against new infections but rather limit their clinical signs. Apart from the bacterin vaccines, subunit vaccines are also an avenue to protect cows against *S. aureus* IMI (Middleton et al., 2009). *Staphylococcus aureus* is a very versatile pathogen that can adhere to and multiply into mammary epithelial cells. It can also multiply in the cittern, and produce biofilms, lytic enzymes as well as molecules to evade the immune system (Côté-Gravel and Malouin, 2019). In a previous study, when single deletion mutants were used to test the importance of specific genes in the pathogenesis of *S. aureus*, an attenuation of the capacity of the mutants to survive in the mammary gland was observed . This incomplete attenuation suggests that blocking one virulence factor is unlikely to be enough to efficiently protect cows. Indeed, subunit vaccines are often composed of more than one antigen. Pujato et al. (2018) evaluated the humoral response of a multicomponent subunit vaccine consisting of three antigens (Clumpimg factor A, fibronectin binding protein A and hemolysin beta). Merrill et al. (2019) evaluated the immune response and protective effect of a vaccine composed of a mixture of staphylococcal surface proteins. Similarly, in the present study, we selected six different *S. aureus* antigens to include in a vaccine. All in all, these six antigens were chosen because multiple *S. aureus* strains commonly expressed them at multiple time points during experimental IMI (Allard et al., 2013). Selection was also focused on expected protein localization (bacterial or extracellular surface) and/or cellular functions.

This study represents, to our knowledge, the first attempt to use antigens expressed specifically during IMI to formulate a vaccine against *S. aureus*. Some subunit vaccines are composed of recombinant proteins (*e.g.*, ClfA, FnBPA, Hla, TRAP) which have been described as virulence factors and are under evaluation (Leitner et al., 2011; Pujato et al., 2018), but there is no guarantee that these factors are essential or strongly expressed during IMI. Some commercially available vaccines are composed of bacterins (StartVac® or TopVac®, Hipra, Spain) that include an inactivated *S. aureus* strain expressing slime-associated antigens (Schukken et al., 2014). While the use of whole bacterins delivers a wide selection of antigens which are useful for developing an adequate immune response, the diversity and type of *S. aureus* strains present in some herds may not necessarily match that of the bacterins used for vaccination. Merrill et al. (2019) evaluated more recently the potential of a mixture of staphylococcal proteins to protect against *S. aureus* IMI. Thus, use of multiple antigens in the formulation of a vaccine has been contemplated by others, but none of these previous vaccines were formulated with antigens known to be important for the bacteria during IMI. The novelty of the present vaccine lies in the selection, as vaccine candidates, of proteins known to be highly expressed during *S. aureus* IMI (Allard et al., 2013).

Six antigens were combined in this vaccine and the amplitude of the immune response against them varied. Although it is tempting to compare the amplitude of the humoral response induced by each antigen, the antibodies may not necessarily have the same affinity or avidity for each antigen, which would create a bias in the analysis. However, calculating the log2 ratio between immune and pre immune serums facilitates the comparison. The differences in the production of total IgG, IgG1 and IgG2 can in part be explained by the following considerations. The same quantity of each antigen was mixed. Because the antigens have different sizes, the number of

molecules of each antigen at the injection site is different. The antigen SACOL0029 is small (8.3 kDa) and it is well known that shorter proteins stimulate less the immune system. To improve the immune response to SACO0029, this antigen could be fused to one of the other antigens. Besides, we opted for the triple adjuvant to tilt the humoral response towards the production of IgG2 that would be very important early in the infection to opsonize the bacteria when their number is still low. Our results showed that in the case of SACOL0442, the IgG2/IgG1 ratio was of 1 which represents a balanced immune response and that our objective was partly obtained for this antigen. It is unclear why we did not observe this for all the antigens. Unfortunately, studies describing the properties of adjuvants are often performed using only one antigen. Ioannou et al (2002) clearly showed an increased IgG2 production against the Bovine Herpes Virus (BHV)-1 glycoprotein D by the addition of the CpG to Emulsigen® D. More work is needed to understand how different antigens stimulate a different response using the same adjuvant.

In this study, we evaluated the CD4+ and CD8+ T cell proliferation induced by the antigens. Comparing our data to those from other studies is difficult because this analysis is rarely performed. In a previous study, we have observed in cows immunized with IsdH (using TiterMax® Gold as adjuvant), that stimulation with that antigen induced CD4+ proliferation. However, in cows immunized with ClfA (also using TiterMax® Gold as adjuvant), no CD4+ or CD8+ proliferation was observed after stimulation with this antigen (Ster et al., 2010). In cows immunized with IsdA and ClfA (fused to the Vibrio cholerae cholera toxinA2/B), a CD4+ cell proliferation but not a CD8+ proliferation was observed when both antigens were used together for stimulation (Misra et al., 2018). In young heifers immunized with two *S. aureus* immune evasion molecules (EfB and LukM) combined to an oil-in-water adjuvant and an alginate hydrogel,

a LukM stimulation tends to induce both CD4+ and CD8+ proliferations while an Efb stimulation tends to induce a CD4+ proliferation (Benedictus et al., 2019). Thus, it is clear that the antigen or more specifically the nature of the epitopes present on the antigen, plays a role in the type of response observed. For instance, with six antigens in the vaccine, we obtained six different responses using the same adjuvant. For example, a small CD8+ cell proliferation was observed for antigen SACOL0720. Besides, with a IgG2/IgG1 ratio of 1, indicative of a higher Th1 immune response, we also could have expected a proliferation of CD8+ T cells by SACOL0442 but this is not what was observed. Unfortunately, studies using similar adjuvant (Ioannou et al., 2002; Kovacs-Nolan et al., 2009) did not present results of CD4+ and CD8+ proliferations. Further studies will be required to better understand how the chosen adjuvant can differently modulate induction of antigen-specific CD8+ effector T cells.

Although careful selection of antigens is of great importance for the development of an effective vaccine, it is also critical to select an appropriate adjuvant. In order to prevent *S. aureus* IMI a vaccine will need to induce a sustained production of IgG, especially IgG2 (Th1 immune pathway or at least a balanced Th1/Th2 response), and also to promote a cellular response (Burton and Erskine, 2003). In this study, we used the combination of Emulsigen®-D, CpG and indolicidin. The combination of Emulsigen®-D and CpG oligodeoxynucleotide (ODN) 2007 was shown to induce a balanced immune response and to provide a higher protection against a BHV-1 challenge in calves; this was better than that obtained with any of the tested adjuvants used alone (Ioannou et al., 2002). CpG nucleotide sequences are immunostimulatory adjuvants and have been shown to promote a Th1 immune response (Mutwiri et al., 2011). In addition, CpG ODN in combination with a cationic antimicrobial peptide (CAMP), such as indolicidin, and polyphosphazene, have

been shown to induce a long lasting Th1 immune response in calves (Kovacs-Nolan et al., 2009). The presence of indolicidin promotes the retention of the antigen at the site of injection (Wilson et al., 2010). An adjuvant capable of inducing a Th1 immune response for a subunit veterinary vaccine is in high demand (Heegaard et al., 2011). The use of multivalent adjuvants, such as that used in this study could be a way to achieve that goal (Mutwiri et al., 2011). Adjuvants and immunomodulation strategies for *S. aureus* vaccines and protection against IMI have recently been reviewed by our group (Côté-Gravel and Malouin, 2019). Future studies could focus on optimizing the proportion of each component in the trivalent adjuvant as well as on the sequence of the CpG (Heegaard et al., 2011) to further direct the immune response against the antigens towards the Th1 pathway and to trigger a sustained production of IgG2.

In this study, we observed low IgG2 titers in milk. It is well known that following an infection, IgG2 will rapidly enter the mammary gland from the blood and that those antibodies will promote opsonisation of the bacteria and their phagocytosis by neutrophils also freshly recruited into the mammary gland (Burton and Erskine, 2003). In this study, the time points selected for detection of IgG2 in milk may not have been optimal and future studies should consider this. IgG2 recruitment is expected to occur early at the onset of the infection (first 24h) when passive transfer of IgG1 is inhibited. Later on, the non specific infusion of IgG1 into the mammary gland is resumed (Burton and Erskine, 2003). Therefore, the established appearance dynamics of IgG1 *vs* IgG2 in the mammary gland is consistent with the high IgG1 *vs* IgG2 titers we were measuring in milk two weeks after the challenge began. It is, however, difficult to compare our results with previous studies evaluating other candidate vaccines as the presence of IgG in the milk is often not given and when provided, the types of data (titers, OD) or sample handling (whole milk, skimmed

milk, whey) are different. However, in other studies, low IgG titers in milk compared to those in serum were also observed following immunization (Boerhout et al., 2015; Camussone et al., 2014; Reidel et al., 2019). Furthermore, Leitner et al (2000) also showed that, in cows chronically infected with *S. aureus,* dilutions necessary for the detection of total IgG, IgG1 or IgG2 in milk are 5 to 30 % of that of blood from the same cow. High level of specific IgG2 flowing quickly into the mammary gland is a requirement for an efficient protection. Ways to promote the local immune response have been proposed. For example, Boerhout et al (2015) showed that immunization routes can have an impact on the immune response and demonstrated that a subcutaneous immunization promotes a higher antibody response against *S. aureus* evasion proteins than that seen with an intramuscular immunization. Although Tomita et al (1998) showed that the localization of the subcutaneous immunization has no impact on the induced immune response; more recently, Boerhout et al (2018), showed that immunizations near the supra-mammary lymph node resulted in higher antibody titers. Further studies are needed to determine the optimal immunization site.

In the present study, we choose to compare the vaccine (antigens plus adjuvants) to saline instead of saline plus adjuvants. The idea was to evaluate the beneficial effects of the vaccine as a whole rather than the effects of one of its components (the antigens). Because the chosen antigens have a high potential for the development of a vaccine, we combined them with an adjuvant cocktail that could help to trigger the appropriate immune response and compared the response of the vaccinated cows to that of naïve cows (saline) after an experimental challenge with *S. aureus*. By doing so, we cannot clearly distinguish the role played by the antigens *vs* the adjuvants in the observed benefits. However, since the challenge was started 4 weeks after the second

immunization a mere stimulation of the innate response by the adjuvant cannot simply explain the differences observed between our two test groups. Indeed, all of the six antigens were capable of inducing a humoral response (total IgG, IgG1 and IgG2) in this study and a negative correlation was observed between *S. aureus* counts over the challenge period and some of the IgG titers for specific antigens prior to the challenge, suggesting the importance of the immune response to such antigens in combating the infection. Such a correlation also provided an idea of the level of antibodies required against each antigen to effectively clear the infection. Altogether, we can hypothesize that the collective immune responses induced by the antigens certainly contribute to the observed benefits of the whole vaccine. This hypothesis will have to be confirmed in future studies that could also include a group of cows treated with the adjuvants alone.

Considering that the antigens used in this study were known to be highly expressed during IMI, and that the vaccination resulted in an immune response against them, a reduction of *S. aureus* counts in the mammary gland could have been expected after the experimental challenge of the vaccinated cows. Unfortunately, no difference in *S. aureus* counts between vaccinated and placebo cows were observed in this study. The number of animals we could use in the biosafety level 2 barn was limited and we therefore opted for an experimental design that infected three of four quarters per animal. This represents a very difficult infectious challenge for the cows to overcome and, even in natural settings, Barkema et al. (2006) have shown that the more quarters that become infected the more difficult the infection can be to tackle. Thus, our experimental design might have rendered very difficult the detection of a significant protective effect. Other researchers have performed challenges using a lower number of infected quarters (Leitner et al., 2003; Watson, 1992) or another method of infection such as teat dipping with a bacterial solution (Merrill et al.,

2019), which in both cases would have represented a lesser challenge to overcome for the immunized cows. Nevertheless, vaccination resulted in a significant reduction of milk SCC. This is indicative of a lower level of inflammation in vaccinated cows and thus, this vaccine is similar to commercially available vaccines which have shown a reduction in clinical signs but no prevention of new IMI (Nickerson et al., 2008; Schukken et al., 2014).

5. Conclusion

Finding an effective vaccine against *S. aureus* IMI remains a challenge. Using antigens known to be specifically expressed by *S. aureus* during IMI may represent a better option to induce a protective immune response. Serum IgG titers measured for some of these antigens inversely correlated with *S. aureus* counts in milk. Also, one antigen generated the desired balanced IgG2/IgG1 response to combat *S. aureus* infections. In this study, although immunization did not protect the vaccinated animals against the robust experimental *S. aureus* infection that was used, a sustained reduction of milk SCC was observed compared to placebo cows. Further optimization of the vaccine may involve adjustments in the adjuvant composition, changes in the immunization schedule and in the route of immunization. The type of experimental challenge also needs be carefully modified to represent more accurately the naturally occurring IMI.

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Table 1: *S. aureus* antigens used for vaccination

Antigen[a]	Function	Predicted localization[b]	Fragment used (amino acid)	Weight (Kda)
SACOL0029	Putative HMG[c]-CoA synthase, partial	C	1-55	8.3
SACOL0442	Putative enterotoxin	E	44-203	19.8
SACOL0720	ABC transporter (VraG)	T	309-455[d]	18.0
SACOL1867	Serine protease (SplC)	E	40-239	22.8
SACOL1912	Glucosamine-6-phosphate isomerase (Csb33)	E	1-199	23.3
SACOL2385	Heat Shock Protein 20 family protein	E or CW	1-142	17.2

[a] The names are from the annotated *S. aureus* COL genome.

[b] Predicted from the PSORTb localization tool (http://www.psort.org/psortb). C, cytoplasmic; E, extracellular; T, transmembrane; CW, cell wall.

[c] Hydroxymethylglutaryl.

[d] The longest extracellular loop of this ABC transporter was used as antigen.

Table 2. Milk total IgG, IgG1 and IgG2 titers against each antigen for the vaccinated and placebo cows six week after the second immunization (10 days after the beginning of the experimental infection).

Antibody titer per antigen	Vaccinated cows[a] (n=9)		Placebo cows[a] (n=10)		P[b]
	Range	Median	Range	Median	
Total IgG titers					
SACOL0029	50-800	200	25-800	25	<0.01
SACOL0442	50-400	200	25-800	200	NS
SACOL0720	50-800	200	25-50	25	<0.0001
SACOL1867	100-800	400	25-1600	200	NS
SACOL1912	200-1600	400	25-100	25	<0.0001
SACOL2385	25-800	200	25-200	25	<0.001
IgG1 titers					
SACOL0029	100-800	200	50-200	100	<0.01
SACOL0442	25-400	25	25-400	25	NS
SACOL0720	100-1600	200	25-25	25	<0.0001
SACOL1867	200-1600	800	25-400	200	<0.05
SACOL1912	100-800	400	25-200	50	<0.0001
SACOL2385	25-800	200	25-50	25	<0.0001
IgG2 titers					
SACOL0029	25-3200	100	25-400	100	NS
SACOL0442	25-200	50	25-200	25	NS
SACOL0720	25-100	25	25-50	25	NS
SACOL1867	25-400	100	25-400	100	NS
SACOL1912	50-3200	200	25-3200	200	NS
SACOL2385	25-1600	200	25-3200	25	NS

[a] Cows were vaccinated twice 10 weeks apart. Vaccinated cows received the mixture of six antigens (SACOL0029, SACOL0442, SACOL0720, SACOL1867, SACOL1912 and SACOL2385) and a trivalent adjuvant (Emulsigen®-D, CpGODN2007 and indolicidin) while placebo cows received saline.

[b] Comparison between vaccinated and placebo cows. NS, not significant.

Table 3. Health parameters for all the cows during the experimental infection

	Body temperature (°C)[1, 2]		Quarter milk appearance[1, 3]		Quarter milk production (kg)[1, 4]	
	Vaccinated cows (n=9)	Placebo cows (n=10)	Vaccinated cows (n=27)	Placebo cows (n=30)	Vaccinated cows (n=9)	Placebo cows (n=10)
d-7	ND[5]	ND[5]	1 ± 0	1 ±0	3.59 ± 0.20[7]	3.63 ± 0.19[7]
d-4	ND[5]	ND[5]	1 ± 0	1 ± 0	3.60 ± 0.20[7]	3.64 ± 0.19[7]
d1[6]	38.3 ± 0.7	38.4 ± 0.2	1 ± 0	1 ± 0	2.91 ± 0.20[7]	3.24 ±0.19[7]
d3	38.7 ± 0.5	39.2 ± 0.7	1.26 ± 0.11	1.43 ± 0.16	2.86 ± 0.14	2.61 ± 0.13
d4	38.6 ± 0.4	38.8 ± 0.3	1.26 ± 0.15	1.93 ± 0.22	2.64 ± 0.14	2.36 ± 0.13
d5	38.3 ± 0.2	38.9 ±0.8	1.40 ± 0.19	2.30 ±0.30	3.10 ± 0.14*	2.38 ± 0.13*
d8	38.4 ± 0.4	38.5 ± 0.5	1.52 ± 0.15	1.93 ± 0.25	2.77 ± 0.14	2.64 ± 0.13
d10	38.3 ± 0.2	38.4 ± 0.2	1.52 ± 0.20	2.06 ± 0.30	2.71 ± 0.14	2.60 ± 0.13
d12	38.4 ± 0.4	38.4 ± 0.1	1.52 ± 0.22	2.03 ± 0.27	2.68 ± 0.14	2.54 ± 0.13
d15	38.6 ± 0.6	38.6 ± 0.4	1.59 ± 0.26	1.53 ± 0.22	2.56 ± 0.14	2.79 ± 0.13
d17	38.4 ± 0.4	38.3 ± 0.3	1.59 ± 0.25	1.33 ± 0.13	2.77 ± 0.14	2.95 ± 0.13
d19	38.4 ± 0.6	38.3 ± 0.2	1.52 ± 0.25	1.50 ± 0.22	2.72 ± 0.14	2.90 ± 0.13
d22	38.4 ± 0.2	38.4 ± 0.2	1.29 ± 0.17	1.43 ± 0.17	2.62 ± 0.14	2.82 ± 0.13
d25	38.4 ± 0.2	38.3 ± 0.3	1.44 ± 0.25	1.30 ± 0.17	2.78 ± 0.14	2.91 ± 0.13
d29	38.3 ± 0.2	38.3 ± 0.3	1.55 ± 0.26	1.37 ± 0.15	2.81 ± 0.14	2.78 ± 0.13
d32	38.6 ± 0.2	38.5 ± 0.3	1.26 ± 0.18	1.30 ± 1.17	2.68 ± 0.14	2.73 ± 0.13
d36	38.8 ± 0.4	38.8 ± 0.4	1.11 ± 0.11	1 ± 0	2.53 ± 0.14	2.70 ± 0.13

[1] Data presented are means ± SEM.
[2] Temperature was measure trice daily.
[3] Foremilk appearance was scored as follows: 1: normal appearance, 2: presence of flakes, 3: presence of small clots, 4: presence of big clots, 5: presence of viscous filaments and watery appearance of the milk, 6: presence of blood.
[4] Milk was harvested using individual quarter milking units at morning milking and weighed for the determination of quarter milk production
[5] Data was not collected before the experimental infection
[6] Experimental infection was performed after the evening milking on d1.
[7] Data prior to the experimental infection were used as covariable for the statistical analysis.
* Statistical difference between vaccinated and placebo cows (P<0.001)

Figures. Ster et al.

Figure 1: Serum total IgG (**A**), IgG1 (**B**) and IgG2 (**C**) titers for the vaccinated and placebo cows. Titers for each of the six antigens included in the vaccine were measured four weeks after the second immunization and immediately prior to the experimental infection. Each symbol represents the titer for one cow (vaccinated cows, n=9, ○; placebo cows, n=10, □). Horizontal lines represent the medians: continuous lines represent the medians for vaccinated cows while dashed lines represent the medians for placebo cows. Statistical analysis compared titers from vaccinated cows to titers from placebo cows: *, P<0.05; **, P<0.01; ****, P<0.0001. When all antigens are considered together: P<0.0001.

Figure 2: Serum IgG2/IgG1 ratios for the vaccinated cows. Titers for each of the six antigens included in the vaccine were measured four weeks after the second immunization. Each symbol represents the ratio IgG2/IgG1 for one cow (n=9). Horizontal lines represent the medians. Statistical analysis compared the ratio of the different antigens to 1: ***, P<0.001; ****, P<0.0001.

Figure 3: Antigen-dependent proliferation of CD4+ cells (**A**) or CD8+ cells (**B**) isolated from the vaccinated and placebo cows. Cells were isolated four weeks after the second immunization and immediately prior to the experimental infection. Each symbol represents the percentage of CD4+ or CD8+ cells that have proliferated for each cow (vaccinated cows, n=9, ○; placebo cows, n=10, □). Horizontal lines represent the medians: continuous lines represent the medians for vaccinated cows while dashed lines represent the medians for placebo cows. The symbol * shows the statistical differences between the vaccinated and the placebo groups: P<0.05. Note: No difference

between the two groups was observed when the same experiment was previously performed before the first immunization except for the antigen SACOL0720 for which a difference for CD4+ cells proliferation was also observed before the first immunization (4.85 % vs 0.07 % for the vaccinated and the placebo cows respectively).

Figure 4: Evolution of *S. aureus* counts (**A**) and somatic cell counts (SCC) (**B**) in the milk of vaccinated and placebo cows. Four weeks and 4 days after the second immunization, 63 CFU of *S. aureus* were infused into three of the four quarters of vaccinated (○) and placebo cows (□) shortly after to the evening milking (day 1, arrow). Aseptic milk samples were taken at morning milking and the viable counts of *S. aureus* (CFU/mL) were determined by 10-fold dilutions and plating on TSA plates while SCC/mL were determined by Valacta (see Materials and Methods section). The continuous line represents data for vaccinated cows while the dashed line represents data for placebo cows. Each symbol represents the means of CFU/mL (in A) or SCC/mL (in B) for all the infected quarters of vaccinated cows (n=27 for vaccinated cows and n=30 for placebo cows). Over the challenge period, milk SSC were lower for vaccinated cows than for placebo cows (***; $P<0.001$).

figure 1

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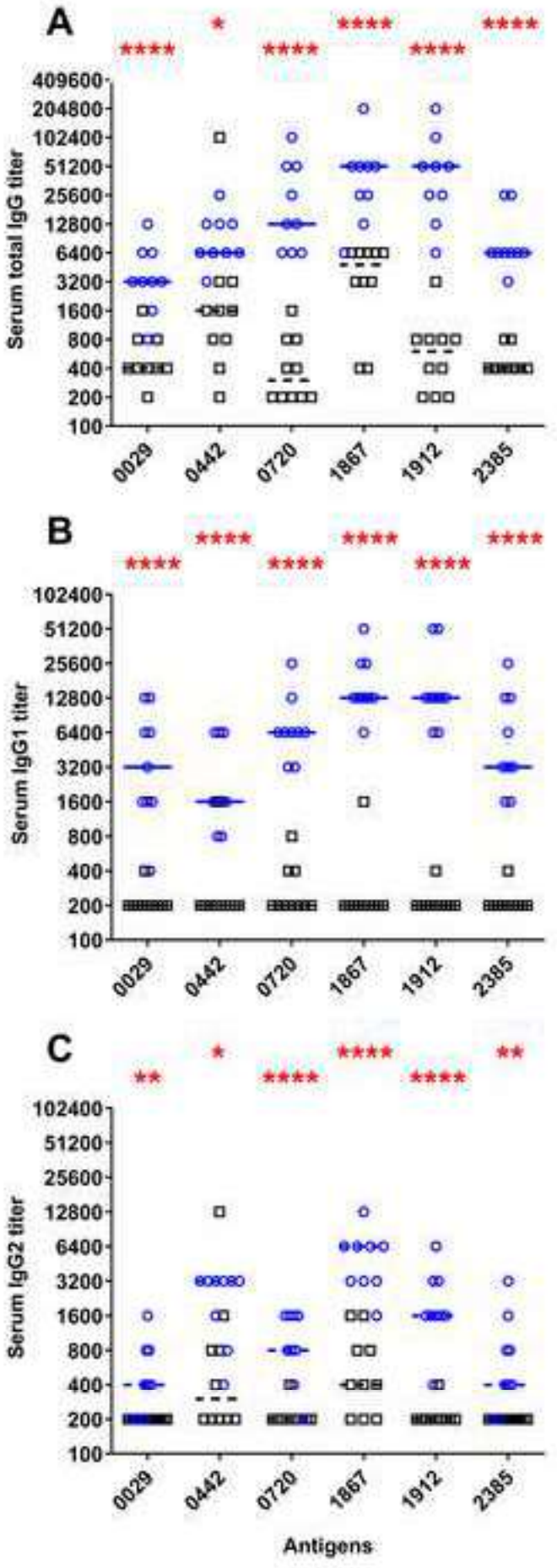

Figure 2

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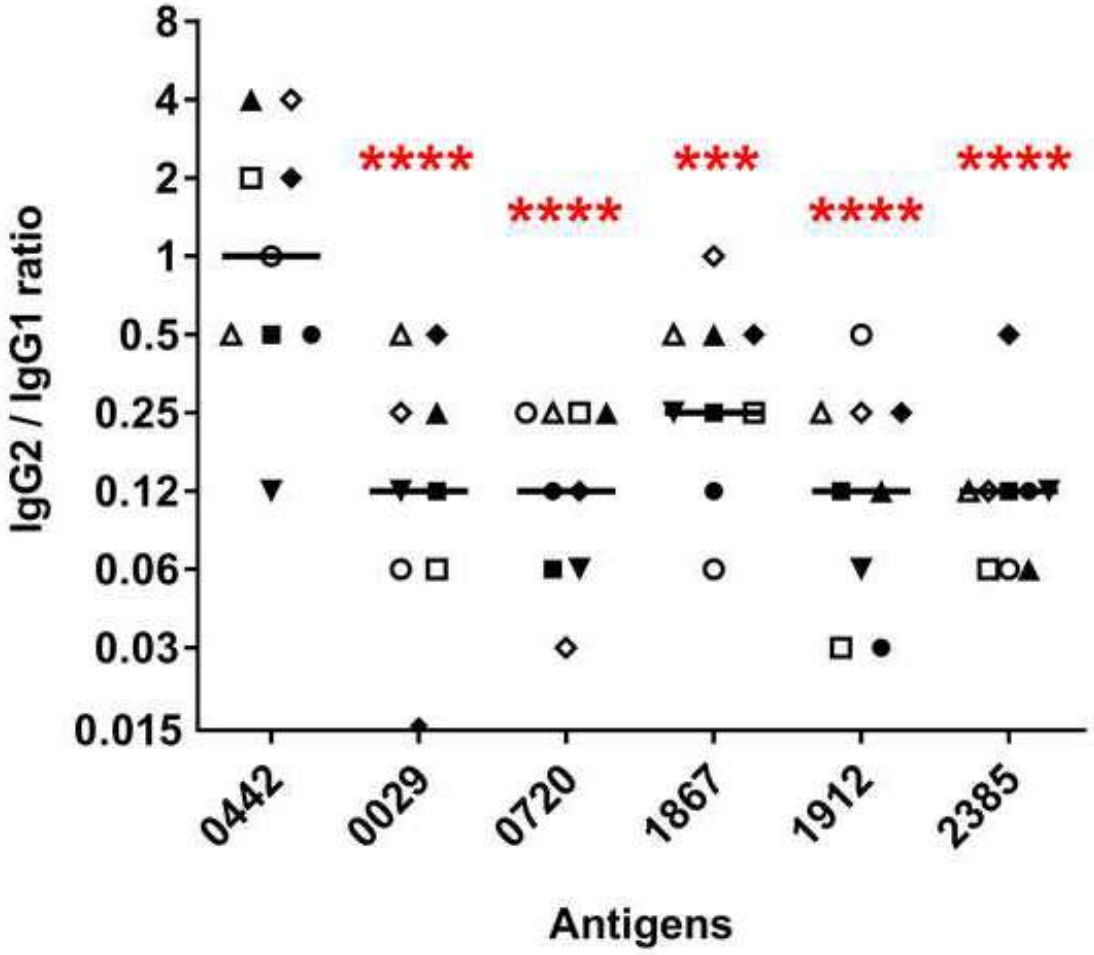

Figure 3

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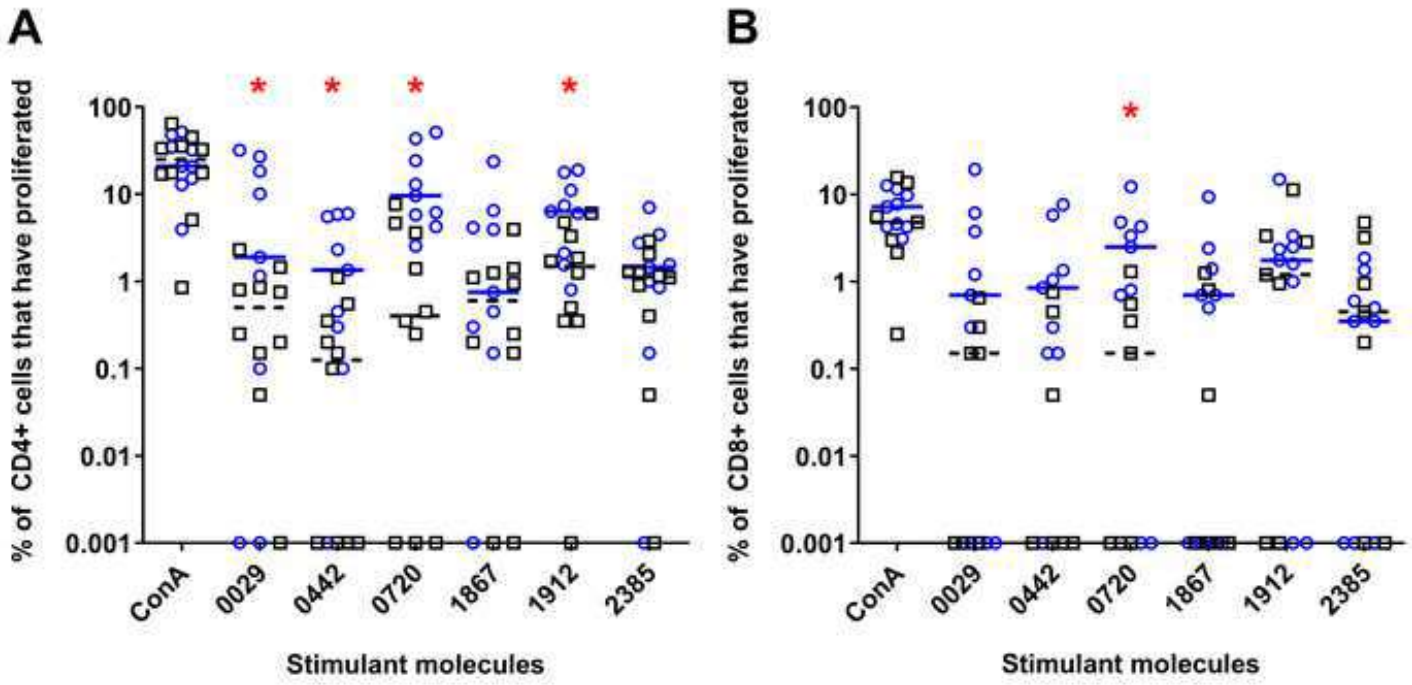

Figure 4

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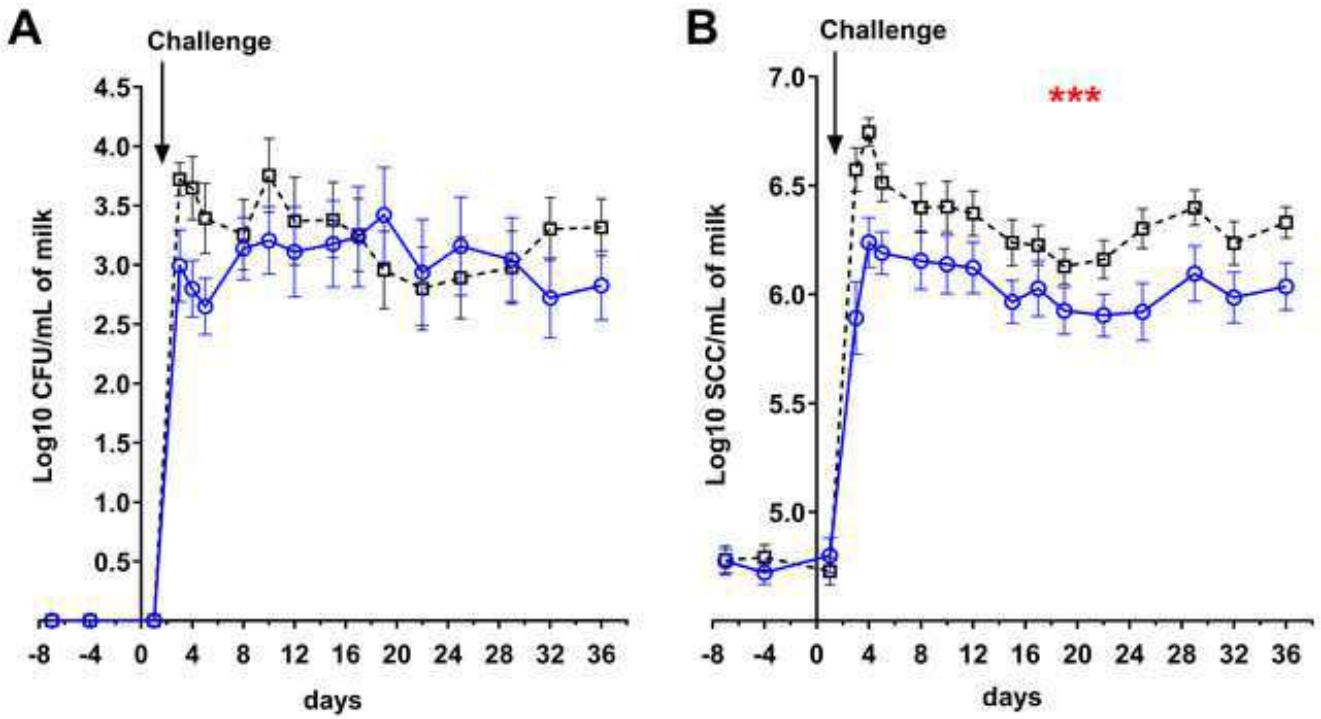

Conflict of Interest

The authors declare no conflict of interest.

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